Developing Methods to Mitigate Chytridiomycosis, an Emerging Disease of Amphibians

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Developing Methods to Mitigate Chytridiomycosis, an Emerging Disease of Amphibians

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Dissertation
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To all the midwife toads that got sampled during this project

"The least I can do is speak out for those who cannot speak for themselves."
- Jane Goodall

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Contents

Zusammenfassung

Amphibien sind stärker gefährdet als irgendeine andere Wirbeltierklasse (Stuart et al., 2004; Hoffmann et al., 2010). Gründe für diese Gefährdung sind vor allem Übernutzung und Habitatsverlust (Stuart et al., 2004). Erst kürzlich tauchte eine weitere Bedrohung auf, die zum Rückgang von Populationen beiträgt und diese sogar zum Aussterben bringt: die sich ausbreitende infektiöse Krankheit Chytridiomykose (Berger et al., 1998; Daszak et al., 2000; Stuart et al., 2004; Skerratt et al., 2007).

Der Infektionserreger der Chytridiomykose ist der Chytridpilz *Batrachochytrium dendrobatidis* (Bd), welcher 1998 entdeckt und 1999 beschrieben wurde (Longcore et al., 1999). Der Pilz gehört zur Abteilung der Chytridiomycota, eine Gruppe von nicht Hyphen bildenden Pilzen (Longcore et al., 1999). Bd kann sich sowohl sexuell als auch asexuell fortpflanzen (Schloegel et al., 2012). Während des asexuellen Lebenszyklus bilden sich frei bewegliche infektiöse Zoosporen, welche die Amphibienhaut besiedeln und Thalli bilden, welche sich zu Zoosporangien entwickeln. In diesen Zoosporangien werden neue Zoosporen produziert, welche durch einen Schlauch aus der Haut ins Wasser entlassen werden. Hier können sie einen neuen Wirt infizieren oder ihren bisherigen Wirt wieder infizieren. (Longcore et al., 1999; Berger et al., 2005).

Bd infiziert keratinisierte Teile der Amphibienhaut, was zu einer Verdickung jener Hautschichten führt, die für viele Stoffe undurchlässig sind. Dies beeinträchtigt die osmoregulatorischen Hautfunktion des infizierten Individuums, was zu Dehydration, einem Ungleichgewicht im Elektrolythaushalt und letztendlich zum Tod führen kann (Voyles et al., 2009).

Heute ist man sich einig, dass Chytridiomykose Verursacherin ist von raschen Populationsrückgängen und lokalem Aussterben. Im “Amphibian Conservation Action Plan“ der Weltnaturschutzorganisation IUCN wir Chytridiomykose charakterisiert als “die schlimmste Infektionskrankheit, die je bei Wirbeltieren festgestellt wurde hinsichtlich der Anzahl betroffener Arten und der Fähigkeit, diese Arten zum Aussterben zu bringen“ (Gascon et al., 2007). Es ist daher wichtig, dass die Gemeinschaft der Wissenschaftler und Naturschutzbehörden die Bedrohung durch die Chytridiomykose erkennt und Massnehmen dagegen entwickeln. Allerdings fehlen uns Methoden zur Bekämpfung dieser noch wenig bekannten Krankheit, was bedeutet, dass

hunderte von Amphibienarten demnächst aussterben werden (Stuart et al., 2004). Der Schlüssel für einen erfolgreichen Amphibienschutz ist daher, neben der Verringerung der Übernutzung und der Wiederherstellung von Lebensräumen, die wirksame Bekämpfung der Chytridiomykose.

Bisher wurden drei verschiedene Ansätze als Bekämpfungsmassnahmen gegen Bd formuliert: Ein Ansatz ist, die Menschverursachte Verbreitung von Bd zu stoppen, mittels Kontrollen des Amphibien Welthandels (Fisher and Garner, 2007) und die Verbreitung von Bd auf kleinem Raum zu verhindern mittels der Entwicklung von Standard Biosicherheits Protokollen (Schmidt et al., 2009a). Ein zweiter Ansatz ist das Einrichten von Erhaltungszuchtprogrammen. Dieser Ansatz ist keine Bekämpfungsmassnahme gegen Bd, aber er schützt empfindliche Arten vor dem Krankheitsbedingten Aussterben (Zippel et al., 2011). Der dritte Ansatz ist die Entwicklung von Bekämpfungsmassnahmen gegen Bd.

Dazu wurden vielerlei Ideen vorgeschlagen und experimentell getestet und wertvolle Resultate wurden generiert. Eine Idee war, Mikro Flora der Amphibienhaut und antimikrobielle Peptide mit Bd-hemmender Aktivität zu verwenden, um Chytridiomykose zu bekämpfen (Woodhams et al., 2011). Die meist versprechenden Strategien sind in diesem Zusammenhang Bakterien in den Boden einzusetzen, welche verhindern, dass Bd die Amphibienhaut besiedelt (Muletz et al., 2012). Zudem wurden Bakterien und antimikrobielle Peptide gefunden, welche die Krankheit bei einigen Amphibien unterdrücken (Woodhams et al., 2011). Eine weitere Idee war es, natürliche Feinde von Bd zu finden oder ökologische Faktoren, welche sich ungünstig auf das Pathogen auswirken (Woodhams et al., 2003). In diesem Zusammenhang waren Behandlungen mit erhöhter Temperatur sehr erfolgreich. Wenn Amphibien bei erhöhter Temperatur gehalten werden, kann dies die Infektion drastisch reduzieren oder das Tier sogar ganz von der Infektion befreien (Woodhams et al., 2011). Eine weitere Idee war schlussendlich der Einsatz von chemischen Fungiziden (Woodhams et al., 2011). In diesem Bereich haben Garner et al. (2009a) eine sehr erfolgreiche Behandlung gefunden, mit welcher Kaulquappen mit dem Fungizid Itraconazol von der Bd Infektion befreit wurden.

Was vorerst fehlt, ist eine Strategie mit welcher Bd in natürlichen Lebensräumen bekämpft werden kann. Gut wäre eine Methode, die nicht nur die Bd Infektion reduziert, sondern auch die Übertragung des Pathogens von infizierten auf un-infizierte Tiere. Die meisten der bisher entwickelten Bekämpfungsmethoden wurden im Labor durchgeführt und keine der Methoden erwies sich in natürlichen Lebensräumen als durchführbar und wirksam. Einige der Methoden wurden noch gar nicht im Feld getestet, hätten aber durchaus das Potential, sehr wirksam zu sein. Andere Methoden mögen aber als Bekämpfungsmethode in natürlichen Lebensräumen ungeeignet sein.

Das Problem ist, dass eine Bekämpfungsmethode in natürlichen Lebens-

räumen gewisse Kriterien erfüllen muss: Zuallererst sollte die Methode die Bd Infektion reduzieren und wenn möglich die Übertragung von Bd verhindern und dies nicht nur unter den kontrollierten Bedingungen im Labor, sondern auch im komplexen Netzwerk eines natürlichen Ökosystems. Des Weiteren sollte die Behandlung keine Nebeneffekte haben weder auf Amphibien noch auf nicht-Zielorganismen im Ökosystem. Eine Behandlung sollte zudem einfach in der Anwendung sein, da sie wahrscheinlich in abgelegenen Gebieten angewendet wird. Schlussendlich sollte es möglich sein, eine grosse Anzahl an Tieren zu behandeln, weil wir ganze Populationen oder zumindest Teile einer Population behandeln müssen. Es ist somit wichtig, dass neue Bekämpfungsmethoden entwickelt werden, die erfolgversprechend sind für die Anwendung in natürlichen Populationen. Dies war das Ziel meiner Doktorarbeit: Die Entwicklung von Bekämpfungsmethoden gegen Chytridiomykose in natürlichen Lebensräumen.

Der Ansatz, den ich hierbei verfolgte war einerseits die Anwendung von Fungiziden, andrerseits testete ich ökologische Faktoren, welche für das Pathogen unvorteilhaft sind. Ich wählte dazu ein Vorgehen in drei Schritten: in einem ersten Schritt testete ich verschiedene Methoden im Labor. In einem zweiten Schritt testete ich diese Methoden in halbnatürlichen Teichen (sogenannten Meskosmen). Hierbei analysierte ich den Effekt des Fungizides auf die Infektion mit Bd und auch auf die Übertragung von Bd. Wesentlich war aber vor allem das Fungizid auf Nebeneffekte auf Amphibien und auf Nicht-Zielorganismen zu testen. In einem dritten und letzten Schritt wurden die erfolgreichsten Methoden ausgewählt für die Tests in natürlichen Teichen. Ziel war hier zu prüfen, ob die Bekämpfungsmethoden gegen Bd ihre Wirksamkeit in einem komplexen Ökosystem aufrechterhalten.

Meine Studienart war hauptsächlich die Geburtshelferkröte (*Alytes obstetricans*) weil bekannt ist, dass diese sehr anfällig ist auf Chytridiomykose und weil sie deshalb beachtliche Populationsrückgänge erlitten hat (Bosch et al., 2010). In den meisten Experimenten arbeitete ich mit Amphibien im Kaulquappen Stadium. Grund dafür ist, dass die Infektion bei Kaulquappen auf die keratinhaltigen Mundfelder beschränkt ist, was nicht zum Ausbruch der Krankheit führt. Die Anfälligkeit der Tiere steigt erst an, wenn sich Keratin in der Haut zu bilden beginnt und wenn das Immunsystem geschwächt ist, was beides kurz nach der Metamorphose der Fall ist (Rollins-Smith, 1998). Wenn also die Infektion bei Kaulquappen eliminiert wird, oder zumindest die Intensität der Infektion reduziert wird, könnte das die Mortalität von metamorphosierenden Tieren reduzieren (Briggs et al., 2010; Vredenburg et al., 2010; Tobler and Schmidt, 2010).

In Kapitel 1 beschreibe ich ein Experiment, in welchem ein ökologischer Faktor als Bekämpfungsmassnahme verwendet wurde. Bd ist empfindlich gegen hohe Temperaturen (Piotrowski et al., 2004). Ich setzte Kaulquappen der Geburtshelferkröte niedriger, mittlerer und hoher Temperatur aus und habe dabei herausgefunden, dass Kaulquappen die Infektion verlieren,

wenn sie fünf Tage lang bei mindestens 26°C gehalten werden. Als Schutzmassnahme empfehle ich daher, die Abschirmung der Sonne durch Bäume zu reduzieren und Flachwasserzonen anzulegen, was zu einer Erhöhung der Wassertemperatur führt (Skelly et al., 2002) und sehr wahrscheinlich zu einem Rückgang der Bd Prävalenz. Warme Teiche beschleunigen zudem die Entwicklung der Amphibien Larven, wodurch weniger Tiere im Larvenstadium überwintern (Thiesmeier, 1992), was die Wachstumsrate der Population steigert (Govindarajulu et al., 2005). Warme Teiche könnten somit das Populationswachstum steigern und gleichzeitig die Bd Prävalenz verringern.

In Kapitel 2 verwendete ich chemische Mittel um infizierten Kaulquappen gegen Bd zu behandeln. Dazu hielt ich Kaulquappen der Geburtshelferkröte sieben Tage lang individuell in Lösungen mit jeweils einem der zwei chemischen Mittel General Tonic® oder Virkon Aquatic® . General Tonic® war sehr wirksam in der Reduktion der Infektion während Virkon Aquatic® keinen Effekt auf Bd zeigte. Kapitel 2 zeigt, dass chemische Mittel durchaus verwendet werden können, um Kaulquappen gegen Infektion mit Bd zu behandeln, jedoch sind weitere Experimente nötig, um Nebeneffekte der Mittel auf Amphibien und auf das Ökosystem zu testen.

Das war das Ziel im Kapitel 3: Im dritten Kapitel berichte ich über ein Experiment, in welchem ich halbnatürliche Mesokosmen einrichtete, um die chemischen Mittel in einem etwas weiteren Kontext zu testen. Ich setzte Mollusken, Insekten, Pflanzen, Krebsartige, Plankton und sowohl infizierte, als auch un-infizierte Kaulquapen der Geburtshelferkröte und uninfizierte Kaulquappen anderer Amphibienarten in die Mesokosmen ein. Danach gab ich jeweils eines von drei Mitteln mit fungizider Wirkung (General Tonic® , Virkon Aquatic® oder PIP Pond Plus®) zum Mesokosmos Wasser dazu. Ich prüfte dann, ob die Mittel die Infektion der infizierten Kaulquappen reduzieren und ob sie die Übertragung von Bd von infizierten auf un-infizierte Tiere stoppen. Zudem testete ich, ob die Mittel Nebenwirkungen auf Amphibien, Mollusken oder andere Faktoren im Ökosystem haben. PIP Pond Plus® hatte keinen Effekt auf die Infektion, hatte aber auch keine Nebeneffekte. General Tonic® reduzierte die Infektion und hatte einige kurzfristige Nebeneffekte auf Mollusken und auf die Struktur des Ökosystems wie z. B. Planktondichte. Die Funktion des Ökosystems war nicht messbar beeinträchtigt, z. B. blieb der Abbau von organischem Material unverändert. Virkon Aquatic® hatte keinen Effekt auf Stärke der Infektion oder Prävalenz, aber es blockierte die Übertragung des Pathogens. Virkon Aquatic® verursachte keine kurzfristigen Nebeneffekte auf das Ökosystem, aber längerfristig hatte es einen Effekt auf die Struktur des Ökosystems, was sich in einem Anstieg der Schneckenreproduktion und Planktonzahl niederschlug. Die entscheidende Frage ist jedoch: Was sind die Kosten und was ist der Nutzen der Behandlung? Während der Nutzen für die Amphibien klar zu sein scheint, wissen wir, dass es Kosten gibt, die jedoch viel schwieriger zu quantifizieren sind. In solchen Situationen emp-

fiehlt man daher einen vorsichtigen Ansatz zu wählen, der da wäre, dass chemische Mittel nur dann in natürlichen Lebensräumen verwendet werden, wenn der erwartete Nutzen gross ist und die Kosten wett gemacht werden. Der Nutzen könnte z. B. gross sein bei einem katastrophalen krankheitsbedingten Rückgang einer Population. In Situationen, wo Bd keine katastrophalen Effekte zu haben scheint z. B. Tobler et al. (2012), ist der Gebrauch von chemischen Mitteln nicht zu empfehlen. In simplen Habitatstypen könnten die Kosten einer Behandlung eher tragbar sein, wie z. B. in den Felsschluchten, die von der Mallorkanischen Geburtshelferkröte *Alytes muletensis* besiedelt werden oder in mensch-gemachten Habitaten wie Kiesgruben. In einem nächsten Experiment testete ich die Behandlungsmethoden mit chemischen Mitteln in natürlichen Lebensräumen.

In Kapitel 4 beschreibe ich drei Experimente, in welchen ich die Behandlung mit den chemischen Mitteln Itraconazol und General Tonic® in natürlichen Teichen testete. Ich mischte das Mittel aber nicht ins Teichwasser, sondern fing die Kaulquappen heraus und behandelte diese in Becken neben dem Teich. So wurden Nebeneffekte auf Nicht-Zielorganismen vermieden. Nach der Behandlung wurden die Kaulquappen wieder in ihrem ursprünglichen Teich freigelassen. Diese Prozedur führte ich zuerst in Mesokosmen durch unter der Verwendung von Itraconazol (Garner et al., 2009a; Tobler and Schmidt, 2010). Dabei fand ich heraus, dass die Behandlung während des Larvenstadiums die Infektion senkt und danach über die Metamorphose hinweg auf tiefem Niveau bleibt, was dann eine verringerte Mortalität unter metamorphosierenden Tieren bewirkt. Diese Behandlungsmethode testete ich daraufhin in einem weiteren Experiment in natürlichen Teichen, wobei die Tiere ein einziges Mal behandelt wurden. Im letzten Experiment behandelte ich die Kaulquappen dann monatlich und benutzte das Mittel General Tonic® anstatt Itraconazol. Nach beiden Teich Experimenten habe ich die Infektion der Kaulquappen im Teich ein Jahr lang beobachtet. Dabei zeigte sich, dass eine chemische Behandlung von Kaulquappen in Becken ausserhalb des Teiches, eine kurzfristige Reduktion der Infektion bewirkt. Dieser Effekt verschwand allerdings wieder nach zwei Monaten. Ein Teil der Kaulquappen wurde nicht gefangen und wurde somit auch nicht behandelt. Bei diesen unbehandelten Kaulquappen wurde die Infektion ebenfalls reduziert, was möglicherweise auf die reduzierte Dichte an infizierten Tieren im Teich zurückzuführen ist. Ein weiteres Resultat war, dass die Infektion im Herbst am tiefsten ist, verglichen mit dem Rest des Jahres. Eine Behandlung im Herbst könnte somit ein neuer Ansatz sein, welcher erlaubt, dass die Infektion auf diesem tiefen Herbst-Niveau gehalten wird. Insgesamt zeigen diese Resultate, dass Fungizide die Infektion reduzieren können, dass sie das Pathogen jedoch nicht gänzlich eliminieren. Die Behandlung ist aber wirksam für eine kurze Zeit und könnte somit als Notfallstrategie dienen im Falle eines krankheitsbedingten Massensterbens.

Kapitel 5 ist eine Sammlung von Behandlungen, die nicht wirksam waren.

Das Kapitel beinhaltet Behandlungen mit Itraconazol, antimikrobiellen Peptiden, Bakterien und mit erhöhter Temperatur. Die Behandlungen waren nicht wirksam hinsichtlich erhöhter Überlebenswahrscheinlichkeit. Dennoch sind diese Resultate wichtig, weil sie zukünftige Forschung voran bringen, indem sie die Grenzen und Möglichkeiten bestimmter Methoden dokumentieren. Aus "gescheiterten" Versuchen lernen ist essentiell, weil die Zeit, die wir verlieren, wenn Fehler mehrmals gemacht werden, ist verlorene Zeit, die wir dringend brauchen für die Entwicklung von wirksamen Bekämpfungsmethoden gegen Bd.

Kapitel 6 ist eine Notiz über die Beobachtung, dass sich Bd und Bakterien auf dem resorbierenden Schwanz von metamorphosierenden Tieren anhäufen. Bisher war es schwierig Bd Infektion auf metamorphosierenden Individuen zu entdecken, weil man nicht wusste, welche Hautstellen Bd bei Tieren in diesem Lebensstadium hauptsächlich besiedelt. Die Notiz in Kapitel 6 hilft, Bd auch auf metamorphosierenden Tieren zu finden.

Zusammenfassend vermittelt meine Doktorarbeit Methoden, Bd in natürlichen Teichen zu bekämpfen. Einige der Methoden wurden in natürlichen Teichen getestet. Wir wissen daher, wie diese Methoden im komplexen Netzwerk eines Ökosystems funktionieren. Ein neuer und ökologisch verträglicher Ansatz könnte sein, bei Teichen die Abschirmung durch Bäume zu reduzieren und Flachwasserzonen zu schaffen, was zu einer Erwärmung des Wassers führt. Dies könnte die Prävalenz von Bd reduzieren und zudem eine erhöhte Wachstumsrate der Population bewirken. Eine andere neue Entdeckung war, dass die Übertragung der Krankheit mit Fungiziden gestoppt werden kann.

Neu ist auch, dass auf dem Markt erhältliche Fungizide sowohl die Stärke der Infektion als auch die Prävalenz reduzieren können. Allerdings haben sie Nebeneffekte auf das Ökosystem. Ich würde daher empfehlen, chemische Mittel nur im Falle von katastrophalen krankheitsverursachten Populationsrückgängen einzusetzen. Ich empfehle auch, Kosten und Nutzen einer Behandlung im Vornherein sorgfältig abzuschätzen. Wenn der Nutzen die Kosten nicht klar aufwiegt würde ich von einer Behandlung des Teichwassers mit chemischen Mitteln unbedingt abraten. Dann empfiehlt sich eher, die Tiere zu fangen und ausserhalb des natürlichen Gewässers gruppenweise in Becken zu behandeln. Dies bringt uns zu einem weiteren neuen Ergebnis meiner Doktorarbeit: Das Herausfangen und chemische Behandeln von Kaulquappen in Becken neben dem Teich reduziert nicht nur die Infektion der behandelten, sondern auch die Prävalenz der unbehandelten Kaulquappen, die im Teich geblieben sind. Interessant ist auch die Beobachtung, das die Infektion im Herbst am tiefsten ist. Eine Behandlung im Herbst könnte daher dazu beitragen, die Infektion auf tiefem Niveau zu halten.

Probleme könnten entstehen, wenn sich neue und virulentere Stämme von Bd entwickeln oder einwandern oder wenn sich bei Bd Resistenzen gegen ein Mittel etnwickeln. Umgekehrt können aber auch anfälligen Amphibien-

arten Resistenzen gegen Bd entwickeln.

Etwas ernüchternd ist natürlich, dass Bd hartnäckig bestehen bleibt. Was wir in der Hand haben, sind ein paar Notfallmassnahmen, mit welchen wir das Pathogen über kurze Zeit eindämmen können. Was wir tun können, ist weiterforschen, um Bekämpfungsmethoden zu finden, mit welchen Infektionen mit Bd reduziert und die Übertragung in ganzen Populationen gestoppt werden können. Dies z. B. mittels Impfung, Züchten von antimikrobiellen Hautbakterien und im Wesentlichen auch mittels ganzheitlichen Ansätzen, die auch Wiederherstellung von Habitaten einbeziehen. Was uns bleibt ist Hoffnung; die Hoffnung, dass mit unseren Bekämpfungsmethoden das Aussterben anfälliger Arten vorerst verhindert werden kann so dass Zeit gewonnen wird, um Bekämpfungsmethoden mit permanenter Wirkung zu entwickeln, so dass Chytridiomykose eines Tages verschwindet von der langen Liste der Bedrohungen der Amphibien.

Summary

Amphibians are more threatened than any other vertebrate taxon (Stuart et al., 2004; Hoffmann et al., 2010). Reasons for this threat are mainly overutilization and habitat loss (Stuart et al., 2004). Only recently a new severe threat to amphibians appeared which contributes to population declines and drives species to extinction: the emerging infectious disease chytridiomycosis (Berger et al., 1998; Daszak et al., 2000; Stuart et al., 2004; Skerratt et al., 2007).

The causative agent of chytridiomycosis is the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd) which has been detected in 1998 and was formally described in 1999 (Longcore et al., 1999). The fungus belongs to the Chytridiomycota, a phylum of non-hyphal fungi (Longcore et al., 1999). Bd can reproduce sexually and asexually (Schloegel et al., 2012). During its asexual life cycle Bd has a motile infective zoospore-stage that colonizes amphibian skin where it forms thalli that develop into zoosporangia. New infective zoospores are produced within the zoosporangia and released through a tube into the water where they can be transmitted to new hosts or re-infect the individual (Longcore et al., 1999; Berger et al., 2005). Bd infects the keratinized amphibian skin inducing thickening of impermeable skin layers which interferes with osmoregulatory functions of the skin and results in dehydration, electrolyte imbalance and eventually in the death of the infected individual (Voyles et al., 2009).

Nowadays it is widely accepted that the amphibian skin disease chytridiomycosis causes rapid population extirpations and declines. In the IUCN Amphibian Conservation Action Plan chytridiomycosis was characterized as "the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (Gascon et al., 2007). It is thus of great importance that the scientific community and conservation agencies recognize and manage the threat of chytridiomycosis. However, the lack of mitigation methods against this poorly understood disease means that hundreds of amphibian species may face extinction (Stuart et al., 2004). Hence, beside habitat restoration and reduction of amphibian exploitation a new key factor in successful amphibian conservation is the effective mitigation of chytridiomycosis.

So far, three different approaches were considered as mitigation strategies

against Bd: One approach is to stop the human induced dissemination of Bd by controlling the world trade in amphibians (Fisher and Garner, 2007) or by developing standard biosafety protocols to avoid spreading Bd on a smaller scale (Schmidt et al., 2009a). A second approach is the establishment of captive breeding programs. This approach is not a mitigation method but it preserves the most susceptible species from disease-induced extinction (Zippel et al., 2011). The third approach is the development of a mitigation strategy against Bd.

Multiple ideas were suggested and tested experimentally as mitigation strategies against Bd and valuable results were achieved: One idea was to use amphibian skin microbiota and antimicrobial peptides with Bd inhibitory activity to combat chytridiomycosis (Woodhams et al., 2011). Here, the most promising strategies are mitigation by introduction of bacteria into the soil which inhibits colonization by Bd on the skin of the amphibian (Muletz et al., 2012). Moreover bacteria and antimicrobial skin peptides were found to suppress disease in some amphibians (Woodhams et al., 2011). A further idea was to find natural enemies of Bd or ecological conditions that are unfavorable for the pathogen (Woodhams et al., 2003). In this context treatments with elevated temperature were very successful. Keeping amphibians in elevated temperature has the potential to clear or drastically reduce infection burdens (Woodhams et al., 2011). Finally, an idea was to use chemical antifungal agents (Woodhams et al., 2011). In this regard a very successful treatment was found by Garner et al. (2009a), who cleared tadpoles with the antifungal agent Itraconazole.

What has been missing until now is a strategy to mitigate Bd in natural habitats potentially including a method which not only reduces Bd infection but also transmission of the pathogen from infected to non-infected individuals. Most of the mitigation methods that were developed so far were conducted in the laboratory and none of them proved to be an implementable and effective strategy to mitigate Bd in natural habitats. Some of these methods were simply not tested yet in natural habitats but have the potential to be very effective. Other strategies might not satisfy the demands of a mitigation method in natural habitats.

Problems could arise because a mitigation method against Bd in natural habitats has to fulfill multiple objectives: First and foremost it should reduce Bd infection and possibly Bd transmission not only under controlled conditions in the laboratory but importantly also in the complex framework of a natural ecosystem. Furthermore a treatment should be safe for amphibians as well as for non-target organisms in the environment. A treatment should also be simple to implement because it might be applied in remote areas. Finally, as we need to treat whole or parts of a population it should be possible to treat large numbers of amphibians. Hence, we need to explore new treatments that seem promising for use in natural populations. This was exactly the goal of my dissertation: Developing methods to mitigate

chytridiomycosis in natural habitats.

The approach I persuaded was on the one hand the use of antifungal agents as a treatment against Bd on the other hand I tested ecological conditions that are unfavorable for the pathogen. In the search for a mitigation method against Bd I followed a three-step approach: in the first step I tested several mitigation methods in the laboratory. In a second step I tested these methods in semi-natural ponds (mesocosms). Here I tested the effect of the antifungal agent on Bd infection and transmission and importantly also side effects on amphibians and non-target organisms. In the third and final step the best methods were selected for tests in natural ponds. The goal was here to verify whether the Bd mitigation methods maintain their effectiveness in the complex framework of a natural ecosystem.

My study species was mainly the midwife toad *Alytes obstetricans* because it is known to be highly susceptible to chytridiomycosis and has suffered substantial declines (Bosch et al., 2010). I used in all the experiments amphibians in the tadpole stage. The reason is that Bd infection in tadpoles is restricted to the keratinized mouthparts, which does not cause disease. Susceptibility to Bd is highest when the skin becomes keratinized and when the amphibian immune system is depressed, which happens both shortly after metamorphosis (Rollins-Smith, 1998). Clearing infection in tadpoles or at least reducing pathogen loads shortly before metamorphosis might thus reduce mortality among metamorphosing individuals (Briggs et al., 2010; Vredenburg et al., 2010; Tobler and Schmidt, 2010).

In chapter 1 I describe an experiment in which an ecological condition was used as mitigation method against Bd. As Bd is sensitive to high temperature (Piotrowski et al., 2004), I exposed tadpoles of *Alytes obstetricans* to low, medium and high temperatures. I found that most tadpoles lost the infection, when they were kept for 5 days at temperatures higher than 26°C. As conservation measures I suggest to reduce canopy cover and to construct shallow water zones which results in the rise of temperatures in the ponds (Skelly et al., 2002) and perhaps prevalence will drop. Additionally warmer water speeds up larval development which implies that fewer tadpoles will hibernate (Thiesmeier, 1992) and therefore population growth rate will increase (Govindarajulu et al., 2005). Warmer ponds may thus boost population growth rate and reduce Bd prevalence. In chapter 2 I used chemical agents to treat infected tadpoles against Bd. I kept tadpoles of *Alytes obstetricans* for seven days individually in solutions of the two chemical agents General Tonic® and Virkon Aquatic® . General Tonic® reduced Bd infection efficiently while Virkon Aquatic® showed no effect on Bd infections. Chapter 2 shows that chemical agents may be used to treat amphibian larvae against Bd infection but further experiments are necessary to test side effects of this agent on the amphibians and the pond ecosystem.

This was the goal in chapter 3. In chapter 3 I describe an experiment in which I set up semi-natural mesocosms to test for the effects of antifun-

gal chemicals in a broader ecological context. I introduced mollusks, insects, plants, crustaceans, plankton and infected and uninfected tadpoles of *Alytes obstetricans* and uninfected tadpoles of other species into the mesocosms. I added three different chemical agents (General Tonic® , Virkon Aquatic® and PIP Pond Plus®) to the mesocosms. I checked for the efficiency of these compounds in reducing Bd infection of infected tadpoles and transmission from infected to uninfected tadpoles. Furthermore, I also tested for side effects of the antifungal treatment on amphibians, mollusks and on different responses of the ecosystem. PIP Pond Plus® had no effect on Bd infections and I did not detect side effects. General Tonic® reduced Bd infection and had some negative short-term effects on mollusks and on the structure of the ecosystem like plankton density. Function of the ecosystem was unaffected returning no effects of the agent on litter breakdown. Virkon Aquatic® had no effect on Bd loads and Bd prevalence of infected individuals, but it blocked Bd transmission. Virkon Aquatic® caused no short-term side effects on the ecosystem, but in the long run ecosystem structure was affected by Virkon Aquatic® , returning an increase in snail reproduction and plankton numbers. However, the crucial question is: what are the costs and the benefits? While the benefit for amphibians seems clear, we know that there are costs but the costs are much harder to quantify. In such a situation, a precautionary approach is recommended. That is, one should only use antifungal agents in natural habitats when the expected benefit is large and likely outweighs the costs. A benefit may be large if one is witnessing a catastrophic Bd-induced population decline. In situations, where Bd does not appear to have immediate catastrophic effects, e.g. Tobler et al. (2012), the use of antifungal agents might not be recommended. Moreover costs of antifungal treatments may be more admissible in simple habitat types like the gorges inhabited by the Mallorcan midwife toad *Alytes muletensis* or man-made habitats such as gravel pits. In a next experiment I tested the treatment with antifungal agents in natural habitats.

In chapter 4 I report on three experiments in which I tested chemical antifungal treatments with Itraconazole and General Tonic® in mesocosms and in natural ponds. I did not add the agent directly to the water. Instead I caught tadpoles out and treated them in tanks next to the pond to avoid side effects on non-target organisms. After the treatment tadpoles were released into the pond. I did this procedure first in mesocosms using the chemical agent Itraconazole (Garner et al., 2009a; Tobler and Schmidt, 2010). Here I found that a treatment in the tadpole stage reduced Bd infection. Bd infection stayed on a low level throughout metamorphosis and reduced the mortality of metamorphosed individuals. Subsequently I tested this method in the second experiment in natural ponds by treating tadpoles once with the chemical agent Itraconazole; in a last experiment I treated tadpoles monthly with General Tonic® . Both experiments in ponds were followed by a monitoring of Bd infection over the period of one year. I found that

treating tadpoles with chemical agents outside the pond and releasing them after the treatment reduced Bd infection in the short term. However the effect disappeared after two months. A part of the tadpoles were not caught out and thus not treated. These untreated tadpoles showed as well reduced Bd infection. Finally I found that Bd infection is lowest in fall. A treatment in fall might thus be a new approach in order to keep Bd infection in a low level. These results suggest that antifungal agents can reduce infection but cannot eradicate Bd from natural environments. However, treatments are effective for a short time and thus might serve as an emergency measure in the case of a Bd-induced mass die-off.

Chapter 5 is a conglomeration of treatments that were not considered as successful. The chapter includes treatments with itraconazole, antimicrobial peptides, bacteria, and elevated temperature to treat amphibians against Bd infection. None of these new experimental treatments were considered successful in terms of improving survival; however, these results may advance future research by indicating the limits and potential of the various protocols. Learning from failed treatments' is essential because the time we lose by doing the same failures twice is valuable time we lose in the development of an urgently needed mitigation method against Bd.

Chapter 6 is a notice about the observation that Bd and bacteria accumulate on the reabsorbing tail of metamorphosing animals. So far it was difficult to detect Bd infection on metamorphosing individuals because of lack of information about the position of Bd on the animal during this developmental stage. The notice in chapter 6 helps to find Bd also on metamorphosing individuals.

In conclusion, my dissertation provides methods to mitigate Bd in natural ponds. Some of these methods were tested in natural ponds and we thus know how they work in the complex framework of a natural ecosystem. A new and ecologically compatible approach might be to reduce canopy cover and to create shallow water zones in order to provide warmer water which may result in lower Bd prevalence and higher population growth rate. Another new finding was that transmission can be blocked by adding antifungal agents to the water.

A new findig was, that commercially available antifungal agents have the potential to reduce Bd loads and Bd prevalence although they have a number of side effects on the ecosystem. I thus recommend chemical treatments of ponds exclusively if a catastrophic Bd-induced population decline is foreseeable and I also recommend to careful assess the costs and benefits of such a treatment in advance. If the benefits do not clearly outweigh the costs, I advise against adding the chemicals directly into the pond water. I rather recommend catching animals and treating them outside the pond in groups. This leads us to a further new outcome of my dissertation: catching tadpoles out of a pond and treating them with a chemical agent in tanks next to the pond reduces not only Bd loads of the treated tadpoles but

also prevalence of those tadpoles that remained in the pond and were never treated. Interesting was the observation that Bd infection is lowest in fall. A treatment in fall might thus help to keep Bd infection on a low level.

Problems could arise through development of new and more virulent strains of Bd or through resistances of Bd against the treatments. Resistances however might also be developed among susceptible amphibian species to Bd.

The drawback of course is that Bd will persist. What we have in hand are some emergency strategies to confine the pathogen for a short term on a limited area. What we can do is go on with research to find mitigation strategies that reduce Bd infection and transmission in whole populations, like blocking transmission, vaccination, culturing antimicrobial skin bacteria and importantly also more integrated strategies including habitat restoration. What remains finally is hope; the hope that our mitigation strategies help to avoid extinction of susceptible species so that we get more time to develop mitigation methods against Bd with permanent effects and one day, hopefully, chytridiomycosis disappears from the long list of threats to amphibians.

General Introduction

Pathogens are ubiquitous. We all know the cold viruses that causes a running nose, coughs and aches. And we are all frightened by other pathogens that cause more serious diseases such as AIDS, cholera, typhus, tuberculosis or malaria, which kill millions of people each year. But pathogens affect much more than our own health. Beekeepers are concerned with the global collapse of honey bee colonies associated with the spread of viruses most probably driven by mites that act as a viral reservoir and incubator. As a consequence, farmers are alarmed as the honey bee is economically one of the most important insects, providing crop pollination services (Martin et al., 2012). Cereal growers struggle with virulent and aggressive fungi that attack their crops which now poses a severe threat to the world's wheat supply (Hovmoller et al., 2010; Fisher et al., 2012).

However the importance of diseases should not be limited to threats in agriculture or human health, which was historically the case. Nowadays, the threat of wildlife diseases is taken more seriously because new outbreaks of diseases were recently registered not only among humans, domesticated animals and plants but also among endangered species (Daszak et al., 2000).

This global emergence of infectious diseases raised concerns among scientists, politicians, conservation managers, and the public that global health is deteriorating. A change in the pattern of occurrence and transmission dynamics of infectious diseases was observed, raising concerns among scientists and stimulating intense reflection on the factors involved in this emergence of diseases. Mirroring the recent advancements in disease awareness and research, the term "emerging infectious disease" (EID) was coined by Joshua Lederberg (Drotman, 1998) in the early 1990s. Emerging infectious diseases can be defined as infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range (Morse, 1995). Nowadays it is widely accepted that emerging infectious diseases (EIDs) are one of the most severe threats to biodiversity and wildlife and this threat is even increasing (Daszak et al., 2000, 2001; Greger, 2007; Smith et al., 2009).

What are the reasons for this new emergence of infectious diseases? Diseases have been with us for millennia and pandemics occurred already many times before. Historical records reveal reports of Egyptian plagues 2000

years ago (Gummow, 2010), the black death raged in Asia and entered Sicily in 1347 from where it spread across Europe (Beran, 2008), and the Spanish conquistadors introduced measles and smallpox into America with disastrous consequences for the native populations who were never confronted with that pathogen, i.e. they were nave to it (Gummow, 2010). These pandemics of the past were often linked to human migration across the globe. This movement of people and their animals has resulted in dissemination of exotic pathogens into nave populations and is therefore an important driver for the emergence of diseases (Daszak et al., 2000; Gummow, 2010).

However, human-mediated dispersal is not the only factor that drives the emergence of infectious diseases. Pathogens interact with other driving factors, such as habitat destruction, climate change, overharvesting, invasive species and environmental pollution, enforcing the emergence of infectious diseases and related local and global extinctions (Smith et al., 2009). It is thus a combination of many factors that boosts the emergence of infectious diseases. A good part of these factors have only recently appeared and emerged which might explain the emergence of infectious diseases. A new devastating EID that has appeared recently is the amphibian skin disease chytridiomycosis. The causative agent of chytridiomycosis is the chytrid fungus Batrachochytrium dendrobatidis (hereafter Bd) which was described formally in 1999 by Longcore et al. (1999).

The fungus belongs to the Chytridiomycota, a phylum of non-hyphal fungi (Longcore et al., 1999). Bd can reproduce sexually and asexually (Schloegel et al., 2012). During its asexual life cycle Bd has a motile infective zoospore-stage that colonizes amphibian skin where it forms thalli that develop into zoosporangia. New infective zoospores are produced within the zoosporangia and released through a tube into the water where they can be transmitted to new hosts or re-infect the individual (Longcore et al., 1999; Berger et al., 2005). An infection with Bd induces proliferation of keratinaceous cells (hyperplasia) and the fusion of the keratin layers (keratosis) in the amphibian skin (Kilpatrick et al., 2010). In adult amphibians this thickening of impermeable skin layers interferes with osmoregulatory functions of the skin resulting in dehydration, electrolyte imbalance and eventually in the death of the infected individual (Voyles et al., 2009).

First signs of this disastrous disease were declines in amphibian populations in ecologically pristine areas like the montane tropical rain forests of Central America and Australia (Daszak et al., 1999). Based on long-term data, scientists showed catastrophic amphibian population declines that eventually resulted in the complete loss of amphibian species (Richards et al., 1993; Mahony, 1996; Pounds et al., 1997; Lips, 1998, 1999). The causal link between the disease chytridiomycosis and these population crashes was not obvious from the beginning on. Historical data indicate that declines began in the early 1970s in the United States Puerto Rico and northeastern Australia (Stuart et al., 2004). In 2004 Stuart et al. (2004) reported

that many declines among amphibian populations were due to habitat loss and overutilization while 48% of the declines were defined as "enigmatic declines". These enigmatic decline species are rapidly declining species that have shown dramatic declines, even where suitable habitat remains, for reasons that are not fully explained. Finally, in 2007 Skerratt et al. (2007) presented evidence that chytridiomycosis, is by far the most likely primary cause of most of these enigmatic" declines of frogs.

Nowadays it is widely accepted that the amphibian skin disease chytridiomycosis causes rapid population extirpations and declines. In the IUCN Amphibian Conservation Action Plan chytridiomycosis was characterized as "the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (Gascon et al., 2007). It is thus of great importance that the scientific community and conservation agencies recognize and manage the threat of chytridiomycosis. However, the lack of mitigation methods against this poorly understood disease means that hundreds of amphibian species may face extinction (Stuart et al., 2004). Hence, beside habitat restoration and reduction of amphibian exploitation a new key factor in successful amphibian conservation is the effective mitigation of chytridiomycosis.

Developing mitigation methods is a challenging issue because it requires a deep understanding of the dynamics of the disease. Disease dynamics are affected by dynamics of the host, pathogen, and vectors, which transport the pathogen between hosts. Host, pathogen and vector are embedded within ecological communities, ecosystems and landscapes which gives a complex framework of countless components of which many have an influence on disease dynamics. Finding a mitigation method against a disease in natural habitats requires that we find the components with the biggest impact on the dynamics of the disease so that we can change them.

Important is information about diversity of hosts and vectors, information about pathogen transmission, pathogen spread on a large scale and information about how the pathogen persists at a site. We also need knowledge about the effects of ecological factors on the disease dynamics for instance disease dynamics under different temperatures or effects of natural predators of the pathogen. Thanks to scientific activities and research valuable insights into these features became apparent.

Host diversity was found to be extremely high: Bd is a generalist pathogen and infects keratinized amphibian skin of over 500 species distributed in at least 54 countries (Fisher et al., 2009). But infection does not necessarily cause disease. Some species are resistant to chytridiomycosis and others suffer mass die-offs (Fisher et al., 2009; Kilpatrick et al., 2010). Multiple asymptomatically infected amphibian species serve as vectors and may be implicated in the transmission and spread of the fungus (Daszak et al., 2001; Weldon et al., 2004; Picco and Collins, 2008). Other factors may also be involved in transmission and spread: there is strong evidence that the am-

phibian trade is contributing to the spread of Bd by the global dissemination of infected animals, by introductions of non-native infected individuals into the wild and by the untreated discharge of zoospores into landscape water regimes (Fisher and Garner, 2007). Additionally, Bd is attracted to the keratinous toes of aquatic birds on which they can adhere and even proliferate. This suggests that waterfowl are potential vectors or environmental reservoirs for Bd (Garmyn et al., 2012).

Once the pathogen is transmitted to a new site, it might persist. Even though persistence of this pathogen is currently poorly understood some patterns became apparent: The fungus can survive up to three months in moist sterile river sand and grows on sterile feathers, dead algae and arthropod exoskeletons (Johnson and Speare, 2005). An important reservoir for Bd is the tadpole stage: Because Bd infects only keratinized cells infection in tadpoles is restricted to the keratinized mouthparts, which does not cause disease. One important reservoir for Bd is thus the tadpole stage: A long-lived tadpole stage that can become infected but not succumb to infection also can promote pathogen persistence (Briggs et al., 2010). How long Bd persists at a site is unclear. In some areas it is even suggested to be native (enzootic) (Longo et al., 2010; Tobler et al., 2012). In areas where Bd is enzootic population declines do not necessarily occur but they might have occurred in the past when Bd arrived. At that time population may have declined to lower abundance and remained stationary at a smaller size after Bd became enzootic (Tobler et al., 2012). In areas where Bd is new (i.e. Bd is epizootic) it spreads in a wave-like fashion and causes populations to collapse (Lips et al., 2006; Wake and Vredenburg, 2008). To successfully protect amphibians from Bd we need thus to develop mitigation methods against this pathogen.

This brings us back to the question: how can we protect amphibians from Bd? So far, three different approaches were suggested: One approach is to stop the human induced dissemination of Bd by controlling the world trade in amphibians (Fisher and Garner, 2007) or by developing standard biosafety protocols to avoid spreading Bd on a smaller scale (Schmidt et al., 2009a). A second approach is the establishment of captive breeding programs to preserve the most susceptible species from extinction (Zippel et al., 2011). The third approach is the development of a mitigation strategy against Bd. Bd mitigation is a difficult task considering the enormous diversity of hosts and vectors, the numerous reservoirs for the pathogen as well as the different ways of transmission and spread. Hitherto, different ideas were suggested and tested experimentally: One idea is to use amphibian skin microbiota with Bd inhibitory activity to combat chytridiomycosis (Woodhams et al., 2011). Another idea is to find antimicrobial peptides that make amphibians resistant to Bd (Woodhams et al., 2011). A further idea is to find natural enemies of Bd or ecological conditions that are unfavorable for the pathogen (Woodhams et al., 2003). And finally, an idea is borrowed from agriculture

where fungal diseases of crops are treated using chemical antifungal agents (Woodhams et al., 2011).

Multiple experiments and studies were undertaken to test these strategies resulting in a series of extremely valuable results. To date, it is possible to treat captive amphibians successfully against infection with Bd (Garner et al., 2009a). However, treating amphibian populations in their natural environments remains a challenge (Woodhams et al., 2011). Here is where my dissertation comes in: The utmost goal of my dissertation was the development of a mitigation strategy against Bd in natural habitats. The methods I tested were mostly chemical antifungal agents as well as ecological factors. My study species was mainly the midwife toad Alytes obstetricans because it is known to be highly susceptible to chytridiomycosis and has suffered substantial declines (Bosch et al., 2010).

My dissertation followed a three-step approach: in the first step I tested several mitigation methods in the laboratory. The aim was, to check whether the methods works in principle under controlled conditions and I also tested for side effects on the amphibian. In a second step I tested these methods in semi-natural ponds (mesocosms). Here I was interested in the effectiveness of the mitigation method under controlled yet more natural conditions. This included not only to test whether the method is reducing infection intensity of animals but also transmission of Bd from infected to uninfected individuals. A very important feature were side effects: I tested side effects on amphibians including histology, behavior and body condition as well as side effects on other aquatic organisms by measuring body condition, activity, survival or development of mollusks, insects, crustaceans and plants. An essential part was also testing side effects on the function of the ecosystem like decomposition of organic material, ability to metamorphose or reproduction. In a third and final step the best mitigation methods were selected for tests in natural ponds. The goal was here to verify whether the Bd mitigation methods maintain their effectiveness in the complex framework of a natural ecosystem. An important feature of this last study was the record of ecological variables to draw conclusions on how the mitigation method works under different ecological conditions.

For all the experiments I used amphibians in the tadpole stage. The reason for this approach is that Bd infection in tadpoles is restricted to the keratinized mouthparts, which does not cause disease. Susceptibility to Bd is highest when the skin becomes keratinized and when the amphibian immune system is depressed, which happens both shortly after metamorphosis (Rollins-Smith, 1998). Clearing infection in tadpoles or at least reducing pathogen loads shortly before metamorphosis might thus reduce mortality among metamorphosing individuals (Briggs et al., 2010; Vredenburg et al., 2010; Tobler and Schmidt, 2010). Moreover tadpoles are often better accessible than adult individuals. A further reason is that population models lead us to expect that enhancing juvenile survival should increase popula-

tion viability (Lampo and De Leo, 1998; Hels and Nachman, 2002; Conroy and Brook, 2003).

In chapter 1 I describe an experiment with elevated temperature as mitigation method against Bd. In chapter 2 an experiment was done with chemical agents to treat infected tadpoles against Bd. In chapter 3 I set up semi-natural mesocosms to test for the effects of chemicals in a broader ecological context. In chapter 4 I report on three experiments in which I tested chemical treatments with Itraconazole and General Tonic® in mesocosms and in natural ponds. Chapter 5 is a conglomeration of treatments that were not considered as successful. Chapter 6 is a notice about the observation that Bd and bacteria accumulate on the reabsorbing tail of metamorphosing animals.

Taken together, a mitigation method against Bd in natural habitats has to fulfill multiple objectives: First and foremost, the treatment strategy should reduce Bd infection and possibly reduce transmission of the pathogen from infected to non-infected individuals. Furthermore a treatment should be safe for individual amphibians as well as for non-target organisms in the environment. And last but not least, a treatment is only effective if we apply it, hence it should be simple to implement. The aim of the three-step approach is to select for those methods which meet these criteria. The three-step approach was based on methods that proved to be efficient in simple and controlled systems in the laboratory. With experiments in more complex systems in mesocosms I gained a more differentiated picture of the methods in terms of effectiveness in reducing Bd infection, in terms of transmission as well as of side effects on the ecosystem. In the course of the experiments in natural ponds the methods had to withstand the rigorous demands of a complex ecosystem. Methods that went through this three-step assessment are methods which might be effective strategies to mitigate Bd in natural habitats.

Chapter 1

Elevated temperature clears chytrid fungus infections from tadpoles of the midwife toad, *Alytes obstetricans*

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Abstract The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd) is sensitive to high temperature. Hence, exposing amphibians to high temperature may be a method to clear Bd infection. However, the effect of exposure to elevated temperature has never been tested in larval stages or temperate species. We experimentally exposed tadpoles of the toad *Alytes obstetricans* to low, medium and high temperatures and found that most, but not all, tadpoles lost the infection when exposed to temperatures higher than 26°C for 5 days. Thus, exposure to elevated temperatures can be used to treat tadpoles against Bd infection.

Keywords chytridiomycosis, disease, infection, temperature, treatment.

1.1 Introduction

Pathogenic chytrid fungi can drive host population dynamics(Ibelings et al., 2004). The chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd) causes the amphibian disease chytridiomycosis that was characterized in the IUCN Amphibian Conservation Action Plan as "the worst infectious

disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (Gascon et al., 2007). Chytridiomycosis is thought to be one of the main reasons for the global decline of amphibians (Houlahan et al., 2000, 2001; Stuart et al., 2004; Skerratt et al., 2007; Collins and Crump, 2009).

Because chytridiomycosis can lead to high mortality of amphibians both in captivity and in the field, there is a great need for methods that can be used to treat amphibians both against chytridiomycosis and Bd infection (Young et al., 2007; Pessier, 2008). Antifungal drugs have been used successfully to clear infection with Bd (Nichols and Lamiranda, 2000; Parker et al., 2002; Garner et al., 2009a; Tobler and Schmidt, 2010), but there are concerns that these drugs may have unwanted side effects (Nichols and Lamiranda, 2000; Woodhams et al., 2003; Garner et al., 2009a). For example, when treating tadpoles of the toad *Alytes muletensis* against Bd infection using Itraconazole (De Beule and Van Gestel, 2001), Garner et al. (2009a) reported depigmentation of the tadpoles. Therefore, Garner et al. (2009a) did not generally recommend Itraconazole as an anti-Bd treatment because of suspected hepatotoxicity. However, Tobler and Schmidt (2010) used Itraconazole to treat tadpoles of *Alytes obstetricans* and observed no depigmentation. Furthermore, Young et al. (2007) report that no treatment using antifungal drugs is consistently effective across species. Hence, a treatment against Bd that has no such side effects would be of great benefit.

Because growth and survival of Bd are highly sensitive to high temperature (Piotrowski et al., 2004), Woodhams et al. (2003) and Retallick and Miera (2007) exposed adult frogs to elevated temperature (37°C and 32°C, respectively). After being exposed to elevated temperature, frogs were no longer infected with Bd. Keeping frogs at high temperature may thus be a treatment against Bd infection that may be free of undesirable side effects. The use of elevated temperature to clear Bd infection requires further testing (Berger et al., 2010). Here, we test whether tadpoles can also be treated against Bd infection by keeping them at an elevated temperature. In many species, tadpoles carry infections and may be an intraspecific reservoir (Brunner et al., 2004). Additionally, tadpoles of many species are more easily accessible than adults. Hence, mitigation strategies may focus on tadpoles rather than adults (Lubick, 2010). We used tadpoles of the locally endangered midwife toad (*Alytes obstetricans*) (Schmidt and Zumbach, 2005) in our experiment because the species is known to be highly susceptible to chytridiomycosis and carries Bd infections in many parts of its range, often at high infection prevalence and infection loads (Bosch et al., 2001; Garner et al., 2005; Tobler and Schmidt, 2010).

1.2 Material and Methods

Study site *Alytes obstetricans* tadpoles where caught in a pond near Zunzgen (canton Baselland, Switzerland) with dip nets. The site was selected because we knew that Bd prevalence was high (Tobler and Schmidt, 2010). Standard hygiene recommendations were followed during field work (Schmidt et al., 2009b,a). For transport to the University of Zürich, tadpoles were individually packed in small plastic containers (1.5 L) filled up to two thirds with pond water. Before the experiment began, they were kept indoors at 19°C. Water was changed twice a week, followed by feeding (Spirulina suspended in tap water).

Procedure in the laboratory After capture, and again 6-10 days after the experimental treatments, each tadpole was swabbed with a sterile rayon tipped plain swab with a plastic applicator (Copan, Italy) in the mouthpart to test for infection with the fungus *Batrachochytrium dendrobatidis* (Bd). Metamorphs (post Gosner stage 42Gosner (1960)) were swabbed on each foot and on the belly. Bd DNA extraction and subsequent real-time PCR followed standard protocols (Boyle et al., 2004; Tobler and Schmidt, 2010). We ran each sample twice (Tobler and Schmidt, 2010). If the two results of one sample were different, the analysis was repeated. For the experiment, we only used tadpoles that tested positive for Bd. One tadpole metamorphosed on the penultimate day of the experiment.

Experimental treatment To test if warm water cures Bd infection in tadpoles of *Alytes obstetricans*, we conducted a factorial experiment in which we manipulated temperature. Tadpoles were exposed to one of three different treatments: a "low" temperature treatment with a constant water temperature of 21.4°C, a "medium" temperature treatment with a constant water temperature of 26.2°C, and a "high" temperature treatment. While the cold and warm treatment were kept at a constant temperature over the whole time of the experiment (5 days or 120 hours), tadpoles in the high treatment were treated differently. The containers were kept at approximately 30°C twice for 8 hours, followed by room temperature for 16 hours. Afterwards they were kept at approximately 30°C for 43 hours. Then they were kept at room temperature until the end of the experiment. Thus, in the "high" treatment, tadpoles were exposed to a mean of 29.7°C for 59 hours, while the mean temperature across the 5 days was 27.1°C. Heating was done by placing experimental containers on two different heating mats (a 25 cm by 35 cm 15 watt Thermolux heat basis and a 51.6 cm by 27.4 cm 28 watt Lucky Reptile thermo mat). The "low" treatment was kept at room temperature without heating or cooling. Temperatures in each experimental unit were measured daily.

For the experiment, we used small round plastic containers. Each contained 500 ml tap water and one tadpole each. Tadpoles were fed 2.5 ml of *Spirulina* suspension ($\bar{1}$20 mg dry food) at the beginning of the experiment and after three days. The plastic containers were placed on a laboratory table next to a window with closed shutters. Since water evaporated, we added water after three days to reach the original volume. 10 tadpoles were kept in the "low", 9 tadpoles in the "medium" and 10 tadpoles in the "high" treatment. All tadpoles were assigned randomly to treatments.

After the experiment, all tadpoles and metamorphs that tested positive for Bd were treated using Itraconazole (Garner et al., 2009a; Tobler and Schmidt, 2010) and all survivors released at the site of capture.

Response variables and statistical analysis The response variable for the statistical analyses was the infection status after the experimental treatment (all tadpoles were initially infected). We conducted two tests. First, we used the experimental treatment levels ("low", "medium", "high") as categorical explanatory variables and a Fisher's exact test to test the null hypothesis of no difference among treatment levels. Second, because there was some variation in temperature among experimental units within treatment levels, we used the mean temperature that a tadpole experienced as a continuous explanatory variable in a logistic regression (Dalgaard, 2008). Statistical analyses were done using the program R (RDevelopmentCoreTeam, 2012). Type I error probability was 5%.

1.3 Results

Three tadpoles died in the "medium" and "high" treatments (one and two, respectively). Loss of Bd infection depended on the temperature treatment. At low, medium and high temperature 2, 5 and 7 out of 10, 8 and 8 tadpoles, respectively, lost the Bd infection (proportions: 0.2, 0.625, 0.875, respectively). The difference among treatments was significant (Fisher's exact test: $p = 0.0262$). The logistic regression based on the mean temperature experienced by tadpoles also showed a highly significant decline of the probability to remain infected with increasing temperature ($p = 0.00825$; Fig. 1.1).

1.4 Discussion

Growth of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* is maximal at 17-25 °C (Piotrowski et al., 2004). Based on this knowledge, Woodhams et al. (2003) and Retallick and Miera (2007) showed that adult frogs can clear Bd-infection when kept for some time at high temperature. Our results (Fig.1.1) show that tadpoles also clear infection when kept at

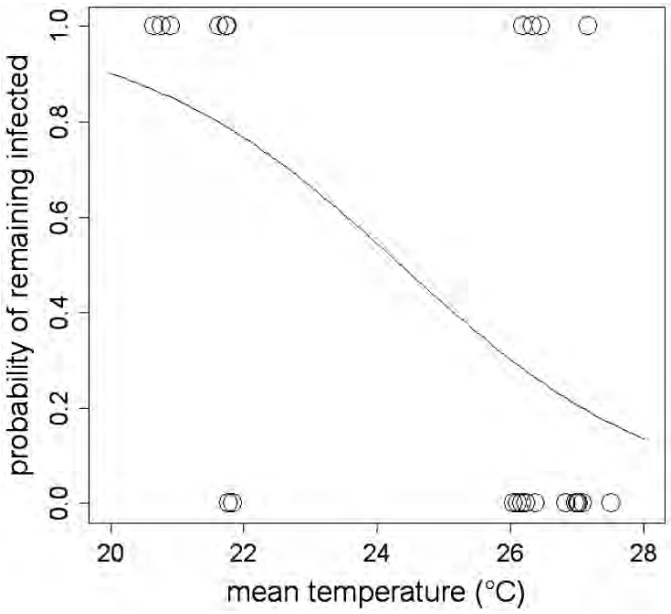

Figure 1.1: Relationship between mean temperature that tadpoles experienced during the experiment and the probability of losing Bd infection. Circles represent individual tadpoles. The equation for the logistic regression line is logit("probability of remaining infected") = 12.356 - 0.507*mean temperature. Standard errors for intercept and mean are 4.797 and 0.192, respectively.

temperatures that are above the fungus' optimal temperature. It may be possible that tadpoles did not clear infection but rather that infection level was below the detection threshold after the treatments. However, Briggs et al. (2010) showed that fungal load dynamics probably explain mortality associated with Bd infection. Hence, even if our treatments only reduced infection loads (as opposed to clearing infection), then this would probably lead to reduced Bd-associated mortality because mortality depends on Bd infection loads (Tobler and Schmidt, 2010).

While there was a clear effect of temperature on the rate of loss of the Bd infection, 2 out of 10 tadpoles that were kept at a mean temperature of 20-21°C also lost the infection. Such a loss of infection was not observed in *Alytes obstetricans* tadpoles in another laboratory experiment where tadpoles were kept at 19°C (Tobler and Schmidt, 2010). A spontaneous loss of infection (and re-infection) was observed, however, in other species (Briggs et al., 2010). Hence, not all losses of infection at higher temperatures observed in our experiment may be due to temperature. Nevertheless, the rate of loss is much higher at elevated temperatures.

Because prolonged exposure to high temperatures cleared most Bd in-

fections, exposure to high temperature may be a simple and inexpensive way to treat large numbers of tadpoles against Bd infections in relatively short time (as it is possible in adult frogs: (Woodhams et al., 2003). We did not measure costs associated with exposure to elevated temperature but we believe that the benefits of the treatment outweigh costs due to side effects. Temperature does not seem to be as efficient (in terms of clearing rates) as antifungal drugs. For instance, Tobler and Schmidt (2010) reported that 100% of the tadpoles were free of Bd after treatment with Itraconazole. Yet, while antifungal drugs may have unwanted side effects (e.g., Garner et al. (2009a)), temperature probably has no such strong side effect because the temperatures that we used are well below the critical thermal maxima reported for tadpoles (usually >38°C; (Ultsch et al., 1999). Hence, one may test the efficacy of higher temperatures or longer exposure times such that Bd infection is eliminated from all tadpoles. Based on the logistic regression equation, less than 1% of the tadpoles is predicted to remain infected at 34°C. When temperature is used to treat tadpoles against Bd infection, we recommend some acclimatization of the tadpoles before the treatment. When novel species are treated, then a pilot study with a small number of tadpoles would be important. Exposure to temperatures that tadpoles occasionally experience in natural ponds ((Thiesmeier, 1992; Indermaur et al., 2010), S. Böll, pers. comm.) cleared most, but not all, Bd infections. It may be possible to use this piece of information to design a mitigation strategy. In some habitats, it may be possible to reduce canopy cover, especially when there are no closed-canopy specialists and when canopy closure is the results of shrub and tree encroachment in the wetland. In a natural experiment where beaver reduced canopy cover, the entire amphibian community benefited (Dalbeck et al., 2007). As a result of reduced canopy cover, temperatures would rise (Skelly et al., 2002) and perhaps prevalence may drop. It may drop to a level that the population may be able to compensate, for example through density dependent processes (Boyce et al., 1999). Such a strategy may have additional benefits in a species with overwintering larvae, such as *Alytes obstetricans*. If warmer water speeds up development such that fewer tadpoles hibernate (Thiesmeier, 1992), then population growth rate is predicted to increase (Govindarajulu et al., 2005). If this reasoning is correct, then warmer ponds may be beneficial for amphibians because Bd prevalence may be lower and population growth rate higher.

Chapter 2

Laboratory tests of antifungal agents

(Diseases of Aquatic Organisms 103: 191-197 DOI:10.3354/dao02576)

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Abstract The fungus *Batrachochytrium dendrobatidis* (hereafter Bd), which is the etiological agent of the disease chytridiomycosis, is threatening amphibians both in nature and in captivity. While there are some methods to treat amphibians in captivity, there is no method yet that has been shown to be a promising treatment for amphibian populations in natural habitats. Here we present the results of an experiment in which we tested two antifungal agents that might be used to treat amphibians in the field. As a first step towards the goal of developing mitigation methods, we tested the efficiency of these agents in reducing Bd prevalence and loads in the laboratory. We exposed naturally infected tadpoles of the midwife toad *Alytes obstetricans* for seven days to different concentrations of the antifungal agents. We found that Virkon Aquatic® had no effect neither on Bd prevalence nor loads (zoospore counts). At 0.625 ml L^{-1} General Tonic® , prevalence was reduced to 60% and infected animals had greatly reduced burdens. However, tadpole length was reduced by 19 % and mass by 32 % on average compared to the control group, suggesting a negative effect on fitness. Survival was not affected at 0.625 ml L^{-1} or 1.25 ml L^{-1} but was reduced to 60 % at 2.5 ml L^{-1} . Keeping animals in a dilution of General Tonic® for seven days at a concentration of 0.625 ml L^{-1} might be an easy way to reduce zoospore counts in large numbers of animals at relatively low costs.

Keywords amphibian, *Alytes obstetricans*, *Batrachochytrium dendrobatidis*, treatment, General Tonic® , mitigation, Virkon Aquatic® .

2.1 Introduction

Newly emerging diseases can pose serious threats to humans, wildlife and biodiversity (Daszak et al., 2000; Jones et al., 2008; Fisher et al., 2012). Even though it is unusual that a pathogen drives its host to extinction, there are some examples where an emerging pathogen caused host extinction (Smith et al., 2009). One of the most fatal diseases for wildlife capable of driving hosts to extinction is chytridiomycosis, a disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd) (Berger et al., 1998; Lips et al., 2006; Skerratt et al., 2007). Chytridiomycosis is now considered a major threat to amphibian species and populations. Hence, successful amphibian conservation depends on effective disease treatment (Woodhams et al., 2011).

While it is possible to treat captive amphibians against infection with Bd, treating amphibians in the wild remains a challenge (Woodhams et al., 2011). A treatment protocol should meet different demands to be successful in the field (Berger et al., 2010; Speare and Young, 2010): 1) a treatment should be highly effective against Bd, 2) a treatment should be effective after a single application, 3) a treatment should act rapidly, 4) as we need to treat whole populations in the field, we need to be able to treat large numbers of amphibians, 5) a method should have a high safety margin, 6) a treatment should be inexpensive, 7) the equipment for a treatment method has to be readily available and 8) a suitable method should cure amphibians in late stage of disease. Most of these criteria are fundamental also for treatments in the laboratory. For a treatment in the field however the number 2) and 4) are essential and also challenging.

Hitherto, multiple attempts were undertaken to find a suitable method to treat amphibians against Bd. This research has resulted in several reports including treatment protocols with elevated temperature, treatments with different antifungal agents, treatment using antifungal skin bacteria or peptides of amphibians, treatment with natural predators or treatment with salt (Woodhams et al., 2011). As it is often the case with antifungal treatments (Denning and Hope, 2010), most of these treatment methods have profound disadvantages because some are toxic for amphibians or not tolerated by them, too labor-intensive for the treatment of large numbers of animals, or some are simply not effective against Bd (Berger et al., 2009; Garner et al., 2009a; Martel et al., 2011; Woodhams et al., 2012). To date, there is no treatment known that fulfills the criteria mentioned above and that was tested successfully in natural habitats. Many methods that could be used for captive amphibians are unlikely to be useful as anti-Bd treatments for amphibian populations in natural habitats as they do not meet the criteria #2 (effective after a single application), #4 (to treat large numbers of amphibians rapidly) and #5 (safe for the environment). Hence, we need to explore new treatments that seem promising for use in natural populations.

Additionally, many treatments were developed to treat adult amphibians. However, in many species, substantial mortality occurs shortly after metamorphosis (Garner et al., 2009b). If one could clear infection in tadpoles or if one could at least reduce pathogen load of tadpoles shortly before metamorphosis, then metamorphosing amphibians would likely survive (Briggs et al., 2010; Vredenburg et al., 2010; Kinney et al., 2011). Hence, there is a need for treatments which can be used for tadpoles.

In the present article, we tested two antifungal agents that could potentially be used for the mitigation of Bd in natural habitats. Virkon Aquatic® (Du Pont, Sudbury, Suffolk, UK) and General Tonic® (Tetra GmbH, Melle, Germany). Both agents were developed to treat large numbers of captive fish against pathogens (including viruses, bacteria and fungi). We chose these agents because the application is very simple even for large numbers of animals and because they are comparatively cheap and widely available. Virkon Aquatic® has the same active substances as Virkon S, a disinfectant that is recommended for the disinfection of field equipment at sites with Bd (Webb et al., 2007) and is not harmful to tadpoles and zooplankton (Schmidt et al., 2009b).

Our study species was the common midwife toad *Alytes obstetricans* which is known to be highly susceptible to chytridiomycosis (Bosch, 2001; Pasmans et al., 2010; Tobler and Schmidt, 2010; Böll et al., 2012). In *Alytes obstetricans* mortality occurs usually shortly after metamorphosis (Tobler and Schmidt 2010). Hence we focused our treatment on the aquatic life stage of the species to make sure that the animals enter metamorphosis with no infection or low infection burden. We treated naturally infected tadpoles of the midwife toad *Alytes obstetricans* with different concentrations of General Tonic® and Virkon Aquatic® .

2.2 Material and Methods

Study site In early March 2011 we collected 176 tadpoles of *Alytes obstetricans* after hibernation from Chalchofen, a site in Switzerland in the canton Baselland (7.76647°E, 47.4775°N, 395 masl). The site is located on a south exposed hillside with sparsely growing trees and bushes around the water bodies. The site consists of four small water bodies of about 3 - 16 m^2 in size and about 0.5 - 0.8 m in depth. Three of the four ponds are located close together while the fourth is at a distance of about 50 m. Two of the four water bodies are densely vegetated while two have no aquatic vegetation. We found tadpoles in one of the two vegetated water bodies as well as in the vegetation-free pond located 50 m away. We chose this site because we knew that i) *Alytes obstetricans* is abundant at that site, ii) tadpoles do hibernate there and iii) amphibians at the site are positive for Bd (in March 2011: n= 176, Bd prevalence = 77%, mean Bd zoospore count ± SE = 96

$\pm$ 10.43).

Procedure in the laboratory We transported the tadpoles to the laboratory and placed them individually in clear plastic 1.5 L-containers with clear plastic covers filled with 1 L of tap water. Holes in the covers enabled circulation of air. Containers were placed on one shelf and randomly allocated to a treatment. Tadpoles were fed *ad libitum* with fish food (Sera Spirulina Tabs, Sera GmbH, Heinsberg, Germany) three times per week throughout the course of the experiment. The water was changed before starting the experiment and thereafter two times per week. The room was equipped with full spectrum sunlight lamps set for a 12 h day length. Room temperature was kept at 19 - 21°C. Six days after capture the tadpoles were staged (Gosner, 1960), weighed to the nearest 0.01 g (Scaletec Instruments, Heiligenstadt, Germany), measured from the snout to the beginning of the tail muscle to the nearest 0.1 mm and swabbed over the mouthparts with a sterile rayon tipped plain swab with a plastic applicator (Copan, Brescia, Italy). Further data on infection, size, and Gosner developmental stage were recorded one week after the experiment was terminated. To avoid cross contamination, gloves were changed between handling different animals, and fresh containers were used for the weighing of each individual. Swabs were analyzed for the presence of Bd with rt-qPCR (see below). Among those tadpoles that tested positive for Bd (136 out of 176), 64 were chosen randomly for use in the experiment. Hence, before starting the experiment Bd prevalence was 100% in all treatment groups.

Antifungal agents We selected Virkon Aquatic® and General Tonic® as possible agents to treat amphibians against Bd. According to the manufacturer, Virkon Aquatic® is an oxygen based disinfectant used for cleaning and disinfection of surfaces associated with aquaculture and has been tested on efficacy against a wide range of fish pathogens including viruses, bacteria and fungi (Antec International and DuPont Animal Health Solutions http://www.antecint.co.uk/MAIN/virkaqua.htm). The active substance in Virkon Aquatic® is potassium peroxomonosulphate triple salt. In the environment it degrades to potassium and sulfate ions. General Tonic® was developed to treat captive fish against bacterial infections, ectoparasites and for treating lesions. According to the manufacturer, the active substances are Ethacridinlactat, Acriflavin, 9-Aminoacridin $*HCl*H_2O$ and methylene blue (Tetra GmbH D 49304 Melle, Germany, http://www.tetra.net).

Experimental treatments Each antifungal agent was tested in 6 different concentrations (including a control). There were 5 replicates for each concentration and 7 replicates for the control. Concentrations were chosen according to recommendations of the manufacturer. Because concentrations

depended on the antifungal agent, we did not treat the experiment as a factorial experiment with the factors "antifungal agent" and "concentration" but treated the tests of the two antifungal agent as separate experiments. For Virkon Aquatic® the concentrations were 1, 2, 3, 4, and 10 mg L^{-1} . For General Tonic® the concentrations were 0.625, 1.25, 2.5, 3.75 and 5 ml L^{-1} . For Virkon Aquatic® the recommended normal concentration for the treatment against different bacteria and viruses is 2 mg L^{-1} while for General Tonic® the recommended normal concentration is 1.25 ml L^{-1} . The different concentrations were achieved by pipetting the corresponding amount of the agent into 1 L of water and stirring it well. Tadpoles were kept individually for 7 days in the corresponding dilution and were observed daily. Animals showing any abnormality in behavior, pigmentation or body shape during the experiment were put into fresh tap water and were removed from the experiment. Dead animals were stored in 98% ethanol. After seven days the water was changed and tadpoles were kept an additional week before swabbing. During this week Bd infection could regenerate after exposure to the agent such that the risk of false negatives could be minimized.

DNA extractions and rt-PCR Upon completion of the experiment, the tips of the swabs were cut off, put in 60 μl of PrepMan Ultra (Applied Biosystems) and extracted applying the bead-beating protocol of Boyle et al. (2004). These extractions were diluted 1/10 and amplified following the rt-PCR protocol of Boyle et al. (2004). Samples were run in duplicate with negative controls and 4 dilutions of standards (100, 10, 1, 0.1 zoospore genomic equivalents, hereafter GE). If the results of the two PCR-wells were inconsistent the analysis was repeated. Values of GE are corrected for the 1/10 dilution.

Response variables and statistical analyses We tested two main features of the antifungal agent: 1) efficiency of the antifungal agent against Bd (taking Bd prevalence and Bd zoospore counts as response variables) and 2) side effects of the antifungal agent on the tadpoles (taking survival and size of the tadpoles as response variables). We measured side effects because we knew from other studies that a treatment can eliminate Bd efficiently but could have unwelcome side effects on amphibians (Garner et al., 2009a; Woodhams et al., 2012).

1) Analysis of efficiency: To analyze differences in prevalence after the treatment among concentrations of the antifungal agent, we used a logistic regression with binomial errors, including prevalence as dependent variable and concentration of the antifungal agent as categorical explanatory variable (recall that prevalence before the treatment was 100%). We also tested for differences in GE after the treatment among concentrations of the antifungal agent using linear regression. In this analysis, the log-transformed GE after

the treatment were dependent variable and the concentration of the antifungal agent was the continuous explanatory variable. Before starting the experiment, GE varied by chance among concentrations of General Tonic® , having higher GE among high concentrations (linear regression with GE as dependent variable and concentration as continuous explanatory variable, p = 0.024). To account for variation in GE at the beginning of the experiment, we included GE before the experiment as covarite in the statistical analysis. We did the analyses separately for General Tonic® and for Virkon Aquatic® .

2) Analysis of side effects on tadpoles: To analyze whether mortality varied among treatments, we used a logistic regression with binomial errors. Survival was the dependent variable and concentration of the antifungal agent the categorical explanatory variable. We tested for an effect of both agents on the size of tadpoles. We used principal component analysis to combine mass and length of the tadpoles after the experiment into a single variable (Schmidt et al. 2012). We used the first principal component axis for further analyses. The first principal component axis (PC1) explained 83% (in the experiment where we used General Tonic® as the antifungal agent) and 96% (in the experiment where we used Virkon Aquatic® as the antifungal agent) of the variance. Both mass and length were positively correlated with PC1 in General Tonic® (r= 0.70 and r= 0.70, respectively) and in Virkon Aquatic® (r= 0.70 and r= 0.70, respectively). We tested for an effect of the antifungal agents on PC1 using a linear regression where PC1 was the dependent variable and the concentration of the agent the categorical explanatory variable. We did both analyses separately for General Tonic® and for Virkon Aquatic® .

All statistical analyses were done in R version 2.15.0 (R Development Core Team 2012).

2.3 Results

Results for treatment with General Tonic® General Tonic® significantly reduced prevalence of Bd (logistic regression, p = 0.018) but did not reduce prevalence to zero (Fig.2.1, Tab.2.1). Prevalence was reduced to 60% at the lowest concentration (Fig. 2.1). Among those that remained infected, tadpoles treated with General Tonic® had significantly lower zoospore counts at all concentrations compared to the control group (linear regression, p = 0.011) (Fig. 2.1, Tab. 2.1).

There was a significant effect of the treatment with General Tonic® on tadpole survival (logistic regression, p = 0.004) (Tab. 2.2, Fig. 2.2). Survival was 100% up to a concentration of 1.25 ml L^{-1} while it was reduced to 60% at 2.5 ml L^{-1} and 0% among 3.75 and 5 ml L^{-1} . Tadpoles started to be lethargic in the General Tonic® treatment at a concentration of 2.5 ml

Table 2.1: Effects of the antifungal agent General Tonic® or Virkon Aquatic® on zoospore counts and on prevalences. At the beginning of the experiment prevalence was 100% in all treatment groups. Prevalence remained 100% among all treatment groups of Virkon Aquatic® . NA indicates that animals were removed from the experiments because of mortality or side effects. There are no p-values for prevalence among those concentrations where prevalence remained 100% (again NA in the table). P-values are for the contrast between control and the respective concentration of the antifungal agent. Significant p-values at $\alpha = 0.05$ are marked with $*$.

		effects on prevalence	effects on zoospore counts
compound	**concentration**	**p-value**	**p-value**
General Tonic®	0 ml L^{-1}	-	-
General Tonic®	0.625 ml L^{-1}	0.122	<0.001*
General Tonic®	1.25 ml L^{-1}	0.026*	0.013*
General Tonic®	2.5 ml L^{-1}	0.005*	0.006*
General Tonic®	3.75 ml L^{-1}	NA	NA
General Tonic®	5 ml L^{-1}	NA	NA
Virkon Aquatic®	0 mg L^{-1}	-	-
Virkon Aquatic®	10 mg L^{-1}	NA	0.872
Virkon Aquatic®	20 mg L^{-1}	NA	0.164
Virkon Aquatic®	30 mg L^{-1}	NA	0.983
Virkon Aquatic®	40 mg L^{-1}	NA	0.598
Virkon Aquatic®	100 mg L^{-1}	NA	0.288

L^{-1} or above. Tadpoles did not exhibit any abnormal behavior at lower concentrations. We did not detect changes of pigmentation. Mass and length of tadpoles, as summarized by PC1, did not differ among treatments at the beginning of the experiment (linear regression, p <0.900). Tadpole size (PC1), was significantly affected by the experimental treatment (linear regression, p = 0.045). An ANOVA, where General Tonic® concentration was treated as categorical explanatory variable, showed that PC1 was significantly lower at all concentrations than in the control (Tab. 2.2).

Results for treatment with Virkon Aquatic® Prevalence of tadpoles treated with Virkon Aquatic® remained 100% at all concentrations. Virkon Aquatic® had no significant effect on zoospore counts (linear regression, p

= 0.577) (Fig. 2.1; Tab. 2.1). Virkon Aquatic® had neither an effect on mortality nor on PC1 (Fig. 2.2; p = 1.0 and p = 0.581, respectively). Survival was 100% for all concentrations. We did neither register any abnormal behavior nor did we find changes in pigmentation.

2.4 Discussion

Outbreaks of chytridiomycosis have led to the extinction of amphibian species and populations (Berger et al. 1998, Bosch et al. 2001, Vredenburg et al. 2010). Therefore, chytridiomycosis is considered to be a major threat to the survival of amphibians (Daszak et al. 2000, Fisher et al. 2012). There are methods to treat amphibians in captivity but most of the published methods cannot be used to treat amphibian populations in natural habitats. For example, Garner et al. (2009a) showed that Itraconazole cleared Bd infection but led to depigmentation of tadpoles. Thus, there is an urgent need to develop a method to treat amphibians in natural habitats against Bd (Woodhams et al., 2011). As a first step towards that goal, we here tested the efficiency of antifungal agents that seemed promising for use in natural habitats. In our experiment we tested whether the antifungal agent General Tonic® and Virkon Aquatic® were effective at clearing infection or reducing infection load and whether they had side effects on tadpoles of *Alytes obstetricans*, a species known to be susceptible to Bd (Bosch et al. 2001, Tobler and Schmidt 2010).

Virkon Aquatic® whose formulation is based on the same active substance as Virkon S which is often used and recommended for the disinfection of field equipment (Webb et al. 2007) did not reduce Bd prevalence or load in individually kept tadpoles in the lab. We exclude it therefore from further discussion and focus on the antifungal agent General Tonic® .

General Tonic® reduced Bd loads efficiently but did not clear all tadpoles totally from Bd (Fig. 2.1). General Tonic® may therefore be used to treat amphibian larvae against Bd infection. It is very easy to apply also for large numbers of animals and at a concentration of 0.625 ml L^{-1} General Tonic® reduced prevalence to 60% and infected animals had greatly reduced pathogen burdens. Survival was not affected at 0.625 ml L^{-1} or 1.25 ml L^{-1} but was reduced to 60% at 2.5 ml L^{-1} . However, General Tonic® should be used carefully because it had an effect on size (PC1) of tadpoles. The long-term consequences of reduced body size on individual fitness (e.g., breeding success) are unknown but should be the focus of further study. Interestingly, the negative effect on PC1 was not detected in an experiment where animals were kept in groups in mesocosms (C. C. Geiger, B. R. Schmidt and F. C. Origgi, unpublished data).

General Tonic® reduced loads but not prevalence. In our opinion, this is an important result because loads determine whether infection leads to

chytridiomycosis and death of individuals. Vredenburg et al. (2010) showed that amphibian mortality begins once infection loads reach a critical threshold of 10,000 zoospores (the threshold may vary among amphibian species). The models of Briggs et al. (2010) and Mitchell et al. (2008) show that reducing Bd infection loads might help populations to persist with endemic Bd infection. Thus, reducing Bd infection loads with General Tonic® could help amphibians to survive despite the presence of Bd. To be helpful in preventing deaths at metamorphosis it would seem that General Tonic® would have to be applied when tadpoles are approaching metamorphosis. We do not claim that General Tonic® is a better treatment than other established and successful agents like for instance itraconazole (Garner et al., 2009a; Woodhams et al., 2011; Brannelly et al., 2012). We selected General Tonic® simply because it might be an agent that could be used as a treatment in the field.

In summary, our experiment demonstrates that General Tonic® may be used to treat amphibian larvae against Bd infection. Future research will include experimental tests of efficiency of General Tonic® as a treatment against Bd infection in mesocosms and natural ponds where side effects of this agent on the amphibians and the pond ecosystem will be tested. These experiments will show whether General Tonic® might be used to treat amphibian populations in natural ponds against the emerging pathogen Bd or whether side effects of this agent are too strong to make it recommendable for application in the environment.

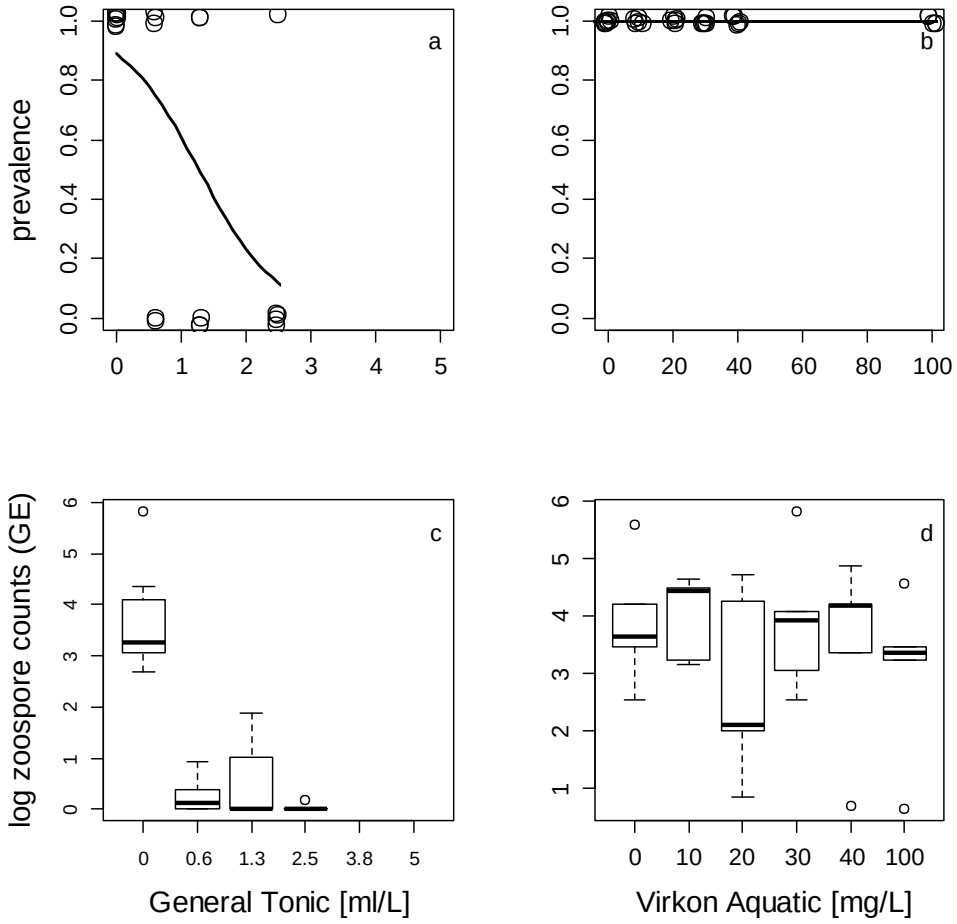

Figure 2.1: a) and b): Effect of different concentrations of General Tonic® and Virkon Aquatic® on prevalence of Bd in tadpoles of *Alytes obstetricans*. In the General Tonic® treatment prevalence was reduced beginning at the lowest concentration tested (0.625 ml L^{-1}) while Virkon Aquatic® showed no effect on prevalence. Symbols are for individual tadpoles and show whether they cleared infection or whether they remained infected. Symbols were slightly offset horizontally and vertically such that all data points are visible. The line shows prevalence as predicted by the logistic regression. c) and d): Boxplots showing infection loads of tadpoles after a 7-day treatment with different concentrations of General Tonic® or Virkon Aquatic® . Concentration "0" are the control groups. General Tonic® reduced zoospore counts beginning at a concentration of 0.625 ml L^{-1} . The black line represents the median, the box represents the interquartile range containing 50% of the values and whiskers mark the 1.5 fold interquartile range. Outliers are marked with circles.

Table 2.2: Side effects of the antifungal agent on tadpoles. We found a mortality effect among tadpoles that were treated with high concentrations of General Tonic® . There was also a significant effect of General Tonic® on PC1 scores. We could not find any side effects among treatment groups with Virkon Aquatic® . NA in the General Tonic® treatment means that animals were removed from the experiments because of mortality or side effects. In the Virkon Aquatic® treatment NA means that all animals survived. P-values are for the contrast between control and the respective concentration of the antifungal agent. Significant p-values at $\alpha = 0.05$ are marked with $*$.

		effects on mortality	effects on fitness (PC1)
compound	**concentration**	**p-value**	**p-value**
General Tonic®	0 ml L^{-1}	NA	NA
General Tonic®	0.625 ml L^{-1}	1	0.008*
General Tonic®	1.25 ml L^{-1}	1	0.009*
General Tonic®	2.5 ml L^{-1}	0.033*	0.04*
General Tonic®	3.75 ml L^{-1}	0.002*	NA
General Tonic®	5 ml L^{-1}	<0.001*	NA
Virkon Aquatic®	0 mg L^{-1}	-	-
Virkon Aquatic®	10 mg L^{-1}	NA	0.691
Virkon Aquatic®	20 mg L^{-1}	NA	0.196
Virkon Aquatic®	30 mg L^{-1}	NA	0.265
Virkon Aquatic®	40 mg L^{-1}	NA	0.182
Virkon Aquatic®	100 mg L^{-1}	NA	0.969

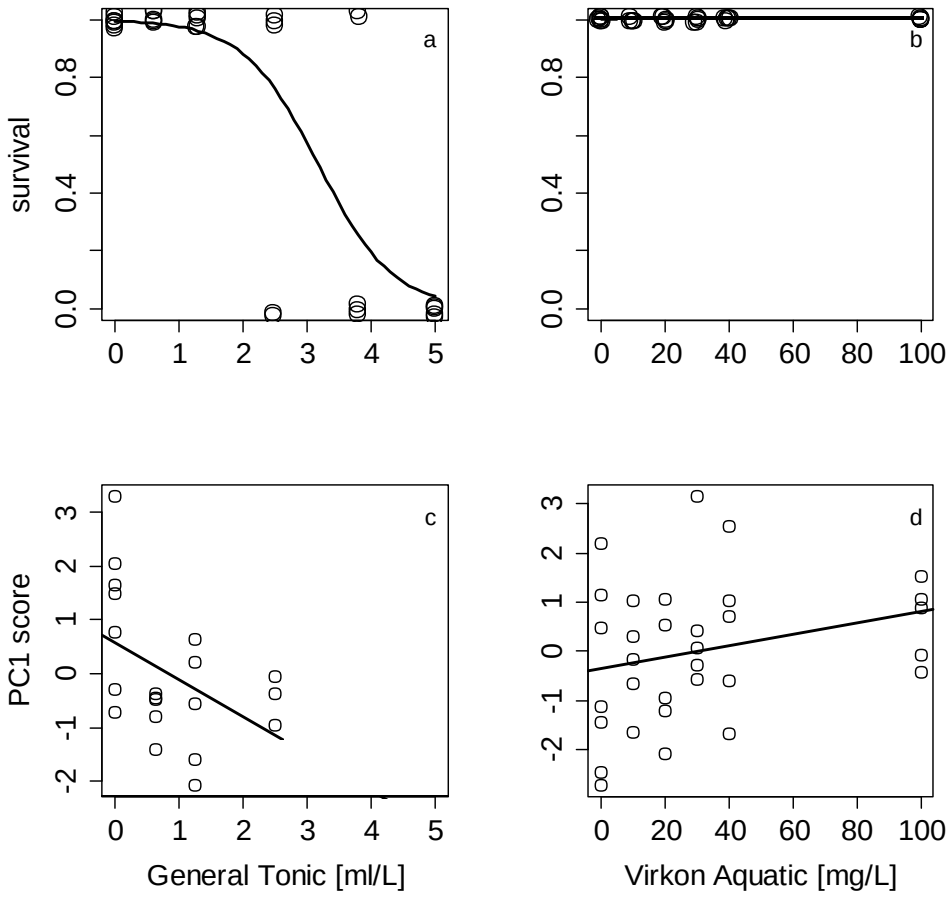

Figure 2.2: a) and b): Effect of General Tonic® on survival of tadpoles. Based on the results of the logistic regression, predicted survival was 100% up to a concentration of 1.25 ml L^{-1} while in Virkon Aquatic® survival was 100% among all concentrations. Symbols are for individual tadpoles and show whether they survived or died. Symbols were slightly offset horizontally and vertically such that all data points are visible. The line shows prevalence as predicted by the logistic regression. c) and d): effect of General Tonic® and Virkon Aquatic® on fitness of tadpoles of *Alytes obstetricans*. Fitness is represented by PC1 scores which are calculated by combining mass and length in a principal component analysis. General Tonic® had a significant negative effect on the PC1 scores while Virkon Aquatic® had no effect on PC1 scores.

Chapter 3

Treating amphibians against chytridiomycosis using antifungal agents: an experimental test of in mesocosms

Corina C. Geiger, Francesco C. Origgi and Benedikt R. Schmidt

Abstract Emerging infectious diseases have been increasing at an alarming rate over the last decades and they might be the key threat to future health of humans and wildlife. Chytridiomycosis, an emerging disease caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd) is causing population declines and substantial loss in amphibian biodiversity. There is no method yet to treat amphibian populations against Bd in natural habitats (ponds). Treating amphibians in their natural habitat means that not only individuals, but whole pond ecosystem may be affected by the antifungal drug. Here, we report on an experiment where we tested whether it is possible to treat larval amphibians against Bd in mesocosms and whether the treatment has side effects on the amphibians and the pond ecosystem. We set up mesocosms and introduced tadpoles of the midwife toad *Alytes obstetricans*, *Bufo bufo*, *Rana temporaria* and other aquatic organisms and we tested three antifungal agents (General Tonic® , Virkon Aquatic® and PIP Pond Plus®). We tested the efficiency of these compounds in reducing Bd infection and transmission. Furthermore, we also tested for side effects of the antifungal treatment on amphibians, snails and on structural and functional metrics of the ecosystem. We found that PIP Pond Plus® had no effect on Bd prevalence or loads, and we also found no noteworthy side

effects. General Tonic® reduced Bd loads, had no effect on prevalence and may reduce Bd transmission. General Tonic® had some negative short-term effects on mollusks and the ecosystem but functional metrics of the ecosystem were unaffected. Virkon Aquatic® had no effect on Bd loads and Bd prevalence of infected individuals, but it blocked Bd transmission. Virkon Aquatic® caused no short-term side effects on the ecosystem. In the long run however, structural attributes of the ecosystem were affected by Virkon Aquatic® , mainly in snail reproduction and in plankton numbers. These results suggest that antifungal agents cannot eradicate Bd from natural environments and have effects on the pond ecosystem. However, the antifungal treatments may be useful as a treatment in the case of a Bd-induced mass die-off. Our results suggest that it may be possible to develop methods to treat amphibians against Bd in their natural environment.

Keywords *Alytes obstetricans*, amphibian, antifungal agent, *Batrachochytrium dendrobatidis*, mesocosm, mitigation, ecosystem structure

3.1 Introduction

Outbreaks of infectious diseases caused by emerging pathogens have increased globally over the past decades. These can pose serious threats to humans, wildlife and biodiversity (Daszak et al., 2000; Jones et al., 2008; Fisher et al., 2012).

Chytridiomycosis is an emerging disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd) (Berger et al., 1998). Chytridiomycosis caused the greatest disease-driven loss in biodiversity ever documented (Fisher et al., 2012) because Bd infects over 500 amphibian species (about 8% of all amphibian species known) in at least 54 countries (27% of all countries globally; (Fisher et al., 2009)). Chytridiomycosis was suggested to be one of the major drivers of the global decline of amphibians (Houlahan et al., 2000; Stuart et al., 2004; Skerratt et al., 2007).

Because chytridiomycosis can drive species to extinction (Berger et al., 1998; Lips et al., 2006; Skerratt et al., 2007), there is an urgent need to learn how to mitigate the negative effects of chytridiomycosis on amphibian populations (Woodhams et al., 2011). While it is possible to treat captive amphibians against infection with Bd, treating amphibian populations in their natural environments remains a challenge (Woodhams et al., 2011). One mitigation strategy is to establish captive breeding programs to preserve the most susceptible species from extinction (Zippel et al., 2011). A second strategy is to find natural enemies of Bd or ecological conditions that are unfavorable for the pathogen (Woodhams et al., 2003). The idea of a third strategy is borrowed from agriculture where fungal diseases of crops are treated using chemical antifungal agents (Woodhams et al., 2011).

A strategy to treat amphibians and to mitigate the effects of chytridiomycosis in the field should fulfill multiple objectives. First and foremost, the treatment strategy should reduce Bd infection loads and prevalence. Furthermore, a treatment should possibly also reduce transmission of infection from infected to non-infected individuals. A further objective is that the treatments should be safe for individual amphibians, i.e. they should have no negative side effects as it was documented for some antifungal agents (Garner et al., 2009a) and is often observed when treating mycoses (Denning and Hope, 2010). Last but not least, a treatment should also be safe for the environment. That is, there should be no side effects on non-target organisms in the aquatic community and ecosystem functions should not be affected. This implies that the effect of antifungal treatment on some measure of "health" of an ecosystem should be assessed. According to Palmer and Febria (2012), the best assessment of ecosystem health is achieved by measuring structural metrics (like diversity of species or turbidity of water) and also functional metrics like reproduction or decomposition rate (Woodward et al., 2012). We designed an experiment with the aim to test the efficiency of anti-Bd treatments using antifungal agents and possible side effects on amphibians and on structural and functional metrics of the aquatic ecosystem.

Here, we report the results of an experiment where we used three antifungal agents to treat larval amphibians against infection with Bd in experimental aquatic ecosystems in outdoor mesocosms (Wilbur, 1997). The three antifungal agents that we used were developed to commercially treat large numbers of captive fish against pathogens (including viruses, bacteria and fungi) and their efficacy in treating Bd has been tested in previous laboratory experiments (Woodhams et al., 2012; Geiger and Schmidt, 2013).

3.2 Material and Methods

Overview

We selected three different amphibian species for our experiment: The common midwife toad *Alytes obstetricans* is known to be susceptible to chytridiomycosis (Bosch, 2001; Tobler and Schmidt, 2010; Böll et al., 2012) and its overwintering tadpoles usually have the highest infection rates in our natural study sites (Tobler and Schmidt, 2010; Böll et al., 2012). Overwintered tadpoles served as infected animals in the experiment (we captured infected individuals). As sentinel species that served to assess transmission of Bd from infected to non-infected individuals, we used tadpoles of *Bufo bufo* (which is susceptible to Bd;(Garner et al., 2009b)), *Rana temporaria* (which is usually not infected; Eliane Küpfer, personal communication) and young of the year *Alytes obstetricans*.

To monitor side effects on other aquatic organisms we added crustaceans

(*Asellus aquaticus*), beetles (*Copelatus* sp.), damselfly larvae (*Coenagrion puella*), snails (*Lymnea* sp.), plants (*Egeria densa*) and different plankton species. We also added leaf litter to the mesocosms to monitor litter breakdown. Leaf litter consisted of beech (*Quercus robur*), oak (*Fagus sylvatica*) and maple (*Acer pseudoplatanus*). Fig. 3.1 gives an overview over the execution of the experiment and the measurements.

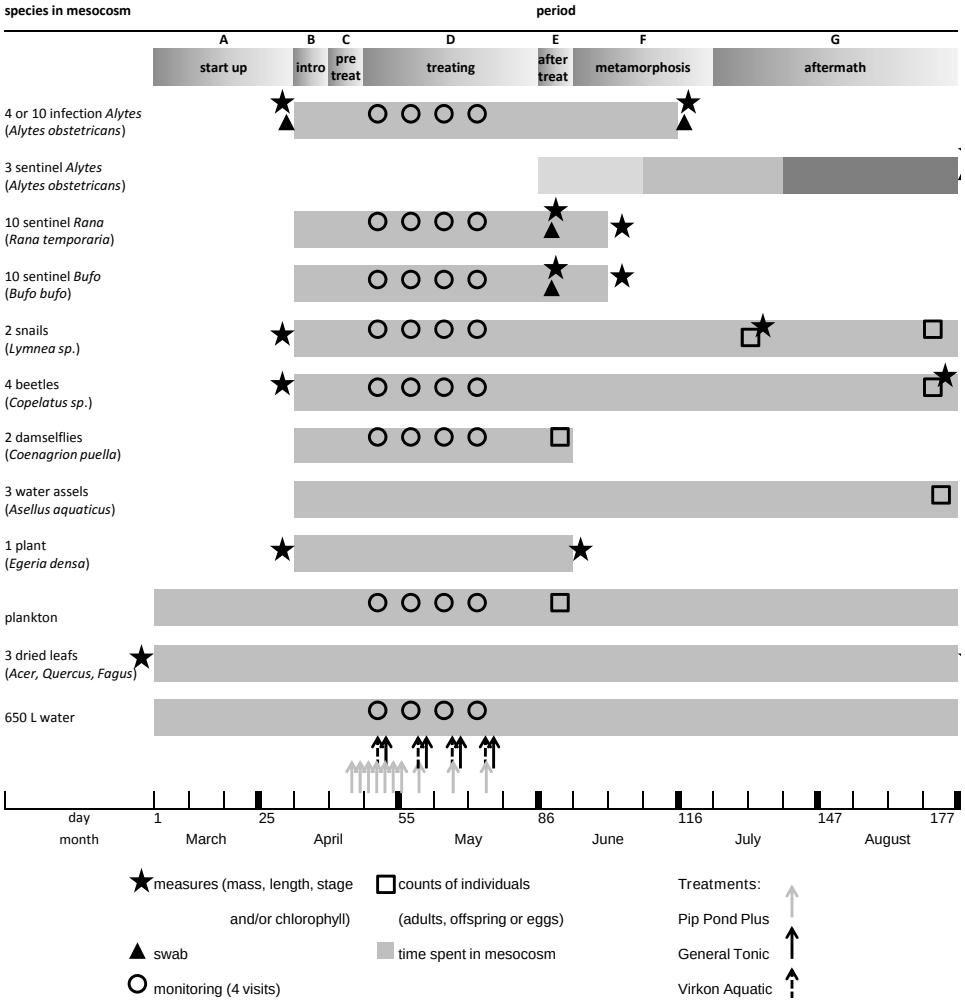

Figure 3.1: Time course of the experiment. The boxes extend from introducing an organism into the mesocosm (left edge) to taking it out (right edge).

Antifungal agents

We chose commercially available antifungal agents that are used in aquaculture. We chose antifungal agents that were easy to apply by simply

adding them to the pond water. We selected three agents for the treatment against Bd: Virkon Aquatic® (Du Pont, Sudbury, Suffolk, UK), General Tonic® (Tetra GmbH D 49304 Melle, Germany) and PIP Pond Plus® (Chrisal, Winters-Van Asten, 3910 Neerpelt, Belgium).

Virkon Aquatic® is an oxygen-based disinfectant used for cleaning and disinfection of surfaces associated with aquaculture and has been tested for efficacy against a wide range of fish pathogens including viruses, bacteria and fungi (Antec International and DuPont Animal Health Solutions; www.antecint.co.uk/MAIN/virkaqua.htm). The active substance in Virkon Aquatic® is potassium peroxomonosulphate triple salt. In the environment it degrades to potassium and sulfate ions. From Webb et al. (2007) we know that Virkon S, whose formulation is based on the same active substance as Virkon Aquatic® , is recommended for the disinfection of field equipment. However in a laboratory experiment, Virkon Aquatic® neither reduced loads nor prevalence of Bd (Geiger and Schmidt, 2013), but effects of Virkon Aquatic® on Bd transmission from infected to non-infected individuals has not yet been tested.

General Tonic® was developed to treat captive fish against bacterial infections, ectoparasites and for treating lesions. The active substances are Ethacridinlactat, Acriflavin, 9-Aminoacridin * HCI * H2O and methylene blue (Tetra GmbH D 49304 Melle, Germany, http://www.tetra.net). In a laboratory experiment, General Tonic® reduced loads and prevalence of Bd (Geiger and Schmidt, 2013). Effects of General Tonic® on Bd transmission from infected to non-infected individuals has not yet been tested. PIP Pond Plus® is a probiotic additive for ponds and fish farms (Chrisal, Winters-Van Asten, 3910 Neerpelt, Belgium; http://www.pippondplus.be/chrisal-de). It was developed to establish a stable microbial community by competitive exclusion of potential pathogenic microorganisms and boosting of the non-pathogenic microbiota. Fish farmers reported less fungal diseases among their fish when PIP Pond Plus® was applied. The exact composition of PIP Pond Plus® is considered a corporate secret by the company but supposed to contain a variety of bacteria and enzymes. In a laboratory experiment, PIP Pond Plus® did neither reduce loads nor prevalence of Bd (Woodhams et al., 2011). Effects of PIP Pond Plus® on Bd transmission from infected to non-infected individuals have not yet been tested.

Mesocosm setup

We set up mesocosms in early March 2010 (on day 1, Fig. 3.1), several weeks prior to the start of the experiment. The experimental units were rectangular fiberglass containers (1.34 m^2, 1000 L volume). We placed these containers outdoors in an open field at the University of Zurich (8.55094°E, 47.39592°N, 507 masl). The mesocosms were started with 650 L of tap water and 400g leaf litter (*Quercus robur*, *Fagus sylvatica* and *Acer pseu-*

doplatanus) as substrate. Mesocosms were covered with green shade cloth to prevent colonization by invertebrates.

We inoculated the mesocosms with several aliquots of pond water with concentrated plankton from a nearby wetland (Bachenbüler Allmend, 8.54295°E, 47.48309°N, 423 masl). In early April 2010 we added a selection of aquatic organisms into each mesocosm. 3 *Asellus aquaticus* (Crustacea; 1 female, 2 males); 4 water beetles (Insecta; *Copelatus* sp.), 2 larvae of damselflies (Insecta; *Coenagrion puella*), 2 water snails (Mollusca; *Lymnea* sp.). All macroinvertebrates were collected in nearby wetlands. All these organisms were freely moving and feeding in the mesocosms except for *Coenagrion puella* which was confined to a floating screen cage of approximately 1 L volume. *Coenagrion puella* fed on plankton that was small enough to enter the cage through the mesh. In addition to these organisms, we introduced one submersed plant of the species *Egeria densa* that we had bought from a store. Furthermore, we placed mesh bags with a mesh width of 1 mm containing dried leaves in each mesocosm. Each mesh bag contained three dried leafs: one of *Quercus robur*, one of *Fagus sylvatica* and one of *Acer pseudoplatanus*. The purpose of these enclosed leaves was to detect side effects of the antifungal agents on the breakdown of organic material by microorganisms. The leaves were weighed at the start of the experiment and at the end.

Experimental design

The experiment had a four-by-two factorial design. The four levels of treatment (the antifungal agents General Tonic® and Virkon Aquatic® , the probiotic agent PIP Pond Plus® and the control) were crossed with two levels of density of infected tadpoles (10 or 4 infected tadpoles of *Alytes obstetricans*). Each treatment x density combination was replicated 3 times while the two control x density combinations were replicated 5 times. Treatments were assigned randomly to mesocosms.

Stocking with amphibians

All amphibians used in the experiment were caught in a pond near Zunzgen, Switzerland (Tobler and Schmidt, 2010). We chose this site because we knew that i) *Alytes obstetricans* is abundant at that site, ii) tadpoles do hibernate there, iii) the site is positive for Bd and iv) the other amphibian species we needed for this experiment occur there too. In early March 2010, we collected 216 tadpoles of *Alytes obstetricans* that had hibernated in the pond. We brought the tadpoles to the university and placed them individually in clear plastic containers. After four days we swabbed every individual over the mouthparts with a sterile rayon tipped plain swab with a plastic applicator (Copan, Brescia, Italy). Swabs were analyzed for the presence

of Bd with rt-PCR (see below). Among those tadpoles that tested positive for Bd, 196 were chosen for the experiment and introduced randomly into the mesocosms in groups of 4 or 10 tadpoles, respectively. We call these tadpoles "infected *Alytes*". When tadpoles entered metamorphosis, infected *Alytes* were caught out of the mesocosms and brought to the laboratory, where they were placed in individual one-liter plastic containers with little tab water and with three dried beech tree (*Fagus sylvatica*) leafs. Containers were tilted so that both land and water were available to the toadlets. The laboratory was equipped with full spectrum sunlight lamps set for a 12 h day length. Room temperature was kept at 19 - 21°C. We fed the toadlets crickets of adequate size ad libitum three times a week. For each individual, the experiment ended when it reached stage 46 or upon death. After the experiment the survivors were treated with Itraconazole and released at their original pond.

We also wanted to test whether intra- and interspecific transmission of Bd from infected to uninfected amphibians is influenced by the treatment with antifungal agents. To test for interspecific transmission of Bd, we introduced 10 laboratory hatched larvae of *Bufo bufo* and 10 larvae of *Rana temporaria* as sentinel tadpoles for interspecific transmission into each mesocosm. Infection with Bd was reported in tadpoles of *Bufo bufo* but not (yet) in *Rana temporaria* tadpoles. To avoid infection of sentinel tadpoles prior to the experiment, we collected eggs in the field and hatched tadpoles in the laboratory. Hatchlings were added to the mesocosms at 3 days of age (*Bufo bufo*) or 8 days of age (*Rana temporaria*). We call these tadpoles "sentinel *Bufo*" and "sentinel *Rana*". After the treatment period 5 tadpoles of sentinel *Bufo* and 5 of sentinel *Rana* were euthanized with MS222 and the remaining 5 of each of them were euthanized upon metamorphosis. To test for intraspecific Bd transmission, we introduced 4 young of the year tadpoles of *Alytes obstetricans* as sentinels two weeks after the last addition of antifungal agents to the mesocosms. We call these tadpoles "sentinel *Alytes*". These sentinel *Alytes* tadpoles stemmed from 10 adult egg string-carrying males of *Alytes obstetricans* that we had collected in Zunzgen. We transported the males in individual boxes to the university, swabbed them over the whole body and analyzed the swabs for the presence of Bd with rt-PCR (see below). As all males turned out Bd-negative, we kept them together in a terrarium of about 0.25m3 containing a land and a water part. Whenever enough tadpoles had hatched to provide each mesocosm with at least one individual, we introduced the tadpoles to the mesocosms (1 tadpole per mesocosm at beginning of June, 1 tadpole at the end of June and 2 tadpoles at the end of July; Fig. 3.1, Tab. 3.1). At the end of the experiment end of August, all sentinel *Alytes* were caught out of the mesocosms, treated with Itraconazole and brought back to their original ponds.

Table 3.1: Measurements taken to evaluate effects of the agents on Bd infection and transmission and to assess side effects of the agents on amphibians, on snails and on structure and function of the ecosystem. See also Fig. 3.1.

species	response	time of measure	measuring method	symbol in Fig.1
Bd infection				
"infected Alytes"	Bd loads, Bd prevalence	A, F	swab (belly, feet)	▲
Bd transmission				
"sentinel *Bufo*"	Bd loads, Bd prevalence	E	individual buccal swab from the mouthparts of 5 tadpoles per mesocosm	▲
"sentinel *Rana*"	Bd loads, Bd prevalence	E	swab (mouthpart of 5 tadpoles per mesocosm)	▲
"sentinel *Alytes*"	Bd loads, Bd prevalence	G	swab (mouthpart of 5 tadpoles per mesocosm)	▲
side effects on amphibian size				
"infection *Alytes*"	PC1 scores of mass and length	A, F	mass: to the nearest 0.01 g length: from the snout to the beginning of the tail muscle to the nearest 0.1 mm	★
"sentinel *Bufo*"	PC1 scores of mass and length	C, E	as above, but no individual measurement for mass (mean among animals in mesocosm)	★
"sentinel *Rana*"	PC1 scores of mass and length	C, E	as above	★
"sentinel *Alytes*"	PC1 scores of mass and length	G	as above	★

side effects on amphibian histology				
"infection *Alytes*"	damages in several organs	F	see "Material and Methods"	★
side effects on amphibian behavior				
"infection *Alytes*"	behavior	D, weekly	counts of tadpoles resting, feeding or swimming during one minute of observation	○
"sentinel *Bufo*"	behavior	D, weekly	as above	○
"sentinel *Rana*"	behavior	D, weekly	as above	○
side effect on snails				
snails (*Lymnea* sp.)	no. clutches, protruding, survival adults	D, weekly	counts of clutches, counts of adults protruding out of the shell, number of surviving adults per mesocosm	○
snails (*Lymnea* sp.)	no. clutches, no. offspring, PC1 scores of mass and length, survival adults	G	counts of egg clutches and offspring on 0.75 m^2 of the mesocosm wall, mass and length of 2 offspring snails per mesocosm and of the 2 adult snails, number of surviving adults per mesocosm	□ ★
short-term effects on ecosystem structure				
beetles (*Copelatus* sp.)	activity	D, weekly	number of swimming beetles monitored within 1 minute of observation	○
damselflies (*Coenagrion puella*)	survival, molting rate	D, weekly	counts of survivors and molts	○
plankton	abundance	D, weekly	1) no, 2) few, 3) normal or 4) clouds of plankton	○
water in mesocosms	turbidity	D, weekly	categories: 1) clear to the bottom, 2) see-through to 40cm depth, 3) to 30 cm, 4) to 10 cm	○

long-term effects on ecosystem structure				
plankton	counts per genus	E	water column of Ø 20 cm and 20 cm depth, filtered with a plankton net (mesh size of 100 µm). The catch was stored in 98% ethanol and individuals per genus were counted under the binocular.	☐
water assels (*Asellus aquaticus*)	counts	G	counts of individuals per mesocosm	☐
beetles *(Copelatus* sp.*)*	mass	C, E	body mass: to the nearest 0.0001 g counts of adults	☐
damselflies (*Coenagrion puella*)	molting rate	E	molting rate estimated from counts of skins found within enclosures,	☐
plants (*Egeria densa*)	chlorophyll content	C, E	mean chlorophyll content of 3 leafs per plant	★
ecosystem function				
littre (*Quercus robur*), (*Fagus sylvatica*) , (*Acer pseudoplatanus*)	littre breakdown	A, G	dry mass before and after the experiment to the nearest 0.0001 g	★

Antifungal treatments

Apart from the control group, we added one of the three different antifungal agents General Tonic® , Virkon Aquatic® or PIP Pond Plus® to each of the mesocosms. The antifungal agents General Tonic® and Virkon Aquatic® were added once a week starting end of April and ending end of May (Fig. 3.1). We added 0.25 mlL^{-1} of General Tonic® per week and 2 mgL^{-1} of Virkon Aquatic® per week. The concentration of Virkon Aquatic® was in accordance with the standard concentration recommended by the manufacturer. In the case of General Tonic® we applied a lower concentration than recommended because in a previous experiment we found negative effects of the recommended concentration on tadpole size (Geiger and Schmidt 2013). The probiotic agent PIP Pond Plus® was applied daily during the first week and weekly during the three following weeks (Fig. 3.1). During the first week, daily concentrations were 0.05 mlL^{-1} water volume, and during the weeks 2, 3 and 4 0.01 mlL^{-1} volume were added weekly. This treatment protocol followed the suggestion of the manufacturer. For all three antifungal agents, the agent was added directly into the mesocosm and mixed by stirring the water for one minute with a plastic stick drawing 8-shaped lines into the water. The stick was dried between uses in different

mesocosms to avoid cross-contamination.

Measurements: We measured five categories of responses: 1) effectiveness of the agents in reducing infection with Bd, 2) transmission of Bd, 3) side effects of the agents on amphibians, 4) side effects on snails and 5) side effects on structure and function of the ecosystem.

Data on the effectiveness of the experimental treatments in reducing Bd infection were measured at metamorphosis (Gosner stage 45) among infected *Alytes*, as tadpoles at the end of the experiment among sentinel *Alytes* and 4 (sentinel *Rana*) or 10 (sentinel *Bufo*) days after the last addition of antifungal agents to mesocosms (Fig. 3.1, Tab. 3.1). All infected *Alytes* were swabbed with a sterile rayon swab over the belly and the feet, as they were already undergoing metamorphosis at that time. Swabs of the sentinel tadpoles were collected from 3 tadpoles (sentinel *Alytes*) or from 5 tadpoles (sentinel *Rana* and sentinel *Bufo*) per mesocosm by collecting individual swabs from the mouthparts with sterile rayon swabs. We analyzed the swabs for the presence of Bd with rt-PCR (see below).

To assess the side effects on amphibians, we recorded the body mass, body length and developmental stage of tadpoles. Infected *Alytes* were measured before they were added to the mesocosms and again at metamorphosis (Gosner stage 45). Sentinel *Alytes* were measured and the development stage was recorded when the experiment was halted on day 177. On day 86 We took the length and defined the stage of 5 tadpoles of sentinel *Rana* and 5 sentinel *Bufo* per mesocosm and measured mass in these groups of 5. Mass was measured to the nearest 0.01g on a Scaletec Scale (Scaletec Instruments, Heiligenstadt, Germany) after blotting tadpoles dry on a paper tissue. Body length was measured to the nearest 0.1 mm from the tip of the snout to the beginning of the tail muscle using a sliding caliper. Developmental stage was based on the Gosner (1960) staging table.

Tadpole behavior was recorded weekly during the month (= 4 visits) when antifungal agents were added to mesocosms by counting in each mesocosm the number of tadpoles (each species separately) resting, feeding or swimming (Van Buskirk 2001). Behavior data was not recorded for sentinel *Alytes*.

To detect effects on internal organs, a subsample of 11 infected *Alytes* per treatment was sacrificed at metamorphosis and then prepared for histological inspection (see below). In order to assess side effects on snails we monitored survival and behavior (retreating into the shell or not) of adult snails and we counted egg clutches weekly during the month (= 4 visits) when antifungal agents were added to mesocosms. On day 129 we registered survival of adult snails. On day 129 and at the end of the experiment we counted the number of egg clutches and the number of offspring on 0.75m2 of the mesocosm wall. Before introduction (adult snails) and on day 129 (adults and offspring) we also measured mass to the nearest 0.01g and shell length to the nearest 0.1 mm of the two adult and of two haphazardly chosen offspring snails in the

mesocosms.

To detect effects on structure of the ecosystem we counted the number of water beetles in each mesocosm weekly during treatment period by recording the swimming beetles detected within one minute of observation. Additionally, before introduction and at the end of the experiment we measured the total mass of beetles per mesocosm with a scale (Mettler Instrumente, Nänikon-Uster, Switzerland) to the nearest 0.0001g, and scored whether reproduction had taken place. For larval damselflies, we counted the number of molted skins per mesocosm (within the enclosure) weekly during the month when antifungal agents were added. We also recorded survival and the number of damselflies that underwent metamorphosis. Effects on crustaceans were recorded by counting the number of crustaceans in every mesocosm at the end of the experiment. The plankton density was monitored weekly during the month when antifungal agents were added to mesocosms as one of the following categories: 1 (no), 2 (few), 3 (intermediate) or 4 (highly abundant). On day 92, we used a plankton net (mesh size 65μm of 19.5 cm diameter) to filter a water column of 20 cm height. From this, we determined the genus of zooplankton present and their respective counts and we counted the number of algae. On day 92, we measured the chlorophyll content of *Egeria* leaves with a chlorophyll meter (Minolta SPAD 502 DL meter, Konica Minolta Photo Imaging, USA). The turbidity of the water in the mesocosms was also assessed on a weekly basis during the treatment as being clear (see-through to the bottom at 50cm depth), see-through to a depth of 40 cm, 30 cm or 10 cm.

Side effects on ecosystem function were assessed by measuring the mass of the beech, oak and maple leaves introduced into the mesocosms with bags before the experiment and at the end on day 177 (Woodward et al. 2012). Dry mass was measured to the nearest 0.0001 g (Mettler Instrumente, Nänikon-Uster, Switzerland).

Histological analysis

A sample of 44 specimens of the infected *Alytes* (11 of each treatment level) was delivered to the Zentrum für Fisch- und Wildtiermedizin (FIWI) in Bern, Switzerland for pathological investigation. There, the tadpoles were fixed in 10% buffered formalin and sectioned cranio-caudally along the midline. The two symmetrical portion of the body were then layered flat in the histocassette, processed as such and embedded in paraffin. Five micron-thick sections were obtained from each specimen and stained routinely with hematoxylin and eosion (H&E). All the sections obtained were screened before full histological examination and additional deep cuts were performed until the major organs were visible in the examined sections, if necessary. Additionally, special stains including Grocott, Gram, PAS, and Zihel-Neelsen were applied as appropriately. Slides were observed under light microscopy

by a single veterinary pathologist and the detected integument changes were recorded for each specimen. Tissue changes affecting any of the observed organs and tissues suggestive of a potential toxic effect were recorded for each individual. Tissue changes were scored as present or absent and subjectively ranked from minimal (+) to severe (+ + ++) by the pathologist. Additionally, changes associated with the presence of fungal organisms and other etiologic agents were also recorded.

DNA extractions and rt-PCR

The tips of Bd swabs were cut off, put in 60 μl of PrepMan Ultra (Applied Biosystems) and extracted applying the bead-beating protocol of Boyle et al. (2004). These extractions were diluted 1/10 and amplified following the rt-PCR protocol by (Boyle et al. 2004). Wells containing 0.01 GE or more were scored positive. Samples were run in duplicate with negative controls and 4 dilutions of standards (100, 10, 1, 0.1 zoospore genomic equivalents, hereafter GE). If the results of the two PCR-wells were inconsistent the analysis was repeated. Values of GE are corrected for the 1/10 dilution.

Response variables and statistical analyses

We tested whether experimental treatments had an effect on Bd infection and prevalence, whether transmission of Bd to uninfected individuals occurred and whether there were side effects on tadpoles and the aquatic ecosystem.

Analyzing effects of antifungal agents on Bd infection loads and prevalence We tested for treatment effects on log-transformed loads of infected *Alytes* individuals using a linear mixed effects model (lme) with antifungal agent and number of infected *Alytes* as explanatory variables. Mesocosm was used as a random variable because individuals within mesocosms were not independent. We deleted one outlier from an infected *Alytes* with extraordinarily high loads that was kept at low tadpole density in the General Tonic® treatment. To analyze differences in prevalence among treatments, we used a generalized linear mixed model (glmer) with a binomial error distribution with infection status of individuals (Bd detected or Bd not detected) as dependent variable and type of antifungal agent and number of infected *Alytes* as explanatory variables. Mesocosm was used as a random variable. Recall that prevalence before the treatment was 100% among the infection *Alytes*. Because we tested two hypotheses on how the agents affect Bd infection, we lowered the usual type I error probability of 0.05 with Bonferroni correction to 0.025.

Analyzing effects of antifungal agents on Bd transmission We tested for differences in log-transformed loads of sentinel *Alytes*, sentinel *Rana* and sentinel *Bufo* among treatments using a linear mixed effects model (lme) with antifungal agent and tadpole density as explanatory variables and mesocosm as grouping (random) variable. To analyze differences in prevalence among treatments, we used a generalized linear mixed model (glmer) with a binomial error distribution with infection status (Bd detected or Bd not detected) as dependent variable and type of antifungal agent, number of infected tadpoles (= tadpole density) and mesocosm as explanatory variables, including mesocosm as grouping (random) variable. We ran this model separately for sentinel *Alytes*, sentinel *Rana* and sentinel *Bufo*. Recall that prevalence before the treatment was 0% among all sentinel tadpoles. In total we tested six hypotheses on how the agents act on Bd transmission. We therefore lowered the usual type I error probability of 0.05 with Bonferroni correction to $0.05/6 = 0.0083$.

Analyzing side effects of antifungal agents on amphibians We tested for an effect of antifungal agents on the size of individual tadpoles. We used principal component analysis to combine mass and length of the metamorphosing animals (infected *Alytes*) or of the sentinel tadpoles (sentinel *Alytes*, sentinel *Rana* and sentinel *Bufo*) after the treatment into a single variable (Schmidt et al. 2012). We took individual measures of infected *Alytes* and of sentinel *Alytes* and the mean of all individuals per mesocosm of sentinel *Rana* and sentinel *Bufo*. The first principal component axis (PC1) explained 90% (infection *Alytes*), 94% (sentinel *Alytes*), 92% (sentinel *Bufo*) and 79% (sentinel *Rana*) of the variance. We used the first principal component for further analyses. We tested for an effect of the antifungal agent on PC1 of infection *Alytes* and sentinel *Alytes* using a linear mixed effects model (lme) where PC1 of either of the two amphibian groups was the dependent variable and antifungal agent and number of infection *Alytes* were the explanatory variables and we included mesocosm as grouping (random) variable. To analyze effect of agent on PC1 (mean values per mesocosm) of sentinel *Rana* and sentinel *Bufo* we used a linear model (lm) where we included PC1 as dependent variable and agent and number of infection *Alytes* as explanatory variables. For detection of effects of agents on histological traits we used a logistic regression with binomial errors, including detected "damages" on the different organs as dependent binomial variable and antifungal agent as categorical explanatory variable.

We tested for differences in behavior of tadpoles among antifungal agents and among tadpole density and among visits using a linear mixed effects model (lme) including the percentage of active tadpoles per mesocosm during one visit as dependent variable and agent, number of infected *Alytes* and visit as explanatory variable and we included mesocosm, as grouping (ran-

dom) variable. We ran the analysis separately for infected *Alytes*, sentinel *Bufo* and for sentinel *Rana* (Tab. 3.3).

Analyzing side effects of antifungal agents on snails Short-term responses of snails measured repeatedly during the month when antifungal agents were added to the mesocosms, were analyzed using MANOVA including the short-term responses of the snails as dependent variable and agent, tadpole density of infected *Alytes* and visit as explanatory variable. We analyzed short-term effects of agent and tadpole density of infected *Alytes* on each response using a linear mixed effects model (lme) including the response of the snails as dependent variable and agent, tadpole density and visit as explanatory variables and we included mesocosm as grouping (random) variable (Tab. 3.4).

Long-term effects of antifungal agents on different features of snails were analyzed using MANOVA where we included the responses of snails measured on day 129 as dependent variables and agent and tadpole density of infected *Alytes* as explanatory variables. To analyze effects of agents and tadpole density on each of these measured responses we fitted linear models (lm) where we included the response of the snail as dependent variable and agent and tadpole density as explanatory variables (Tab. 3.4).

Analyzing side effects of antifungal agents on the aquatic ecosystem We ran three sets of analysis: 1) for detection of short-term effects on structural metrics of the ecosystem including the data we sampled during the 4 visits when antifungal agents were added to mesocosms. 2) Long-term effects on structural metrics of the ecosystem including data of time-point-measurements of different traits measured some weeks or month after the last treatment or at the end of the experiment on day 177. 3) Effects on functional metrics, like litter breakdown and reproduction of snails, measured at the end of the experiment on day 177.

1) Short-term effects on ecosystem structure: We analyzed overall effects of agents and tadpole density on short-term effects using MANOVA where we included responses of ecosystem structure, measured repeatedly during the month when antifungal agents were added to the mesocosms, as dependent variables and agent, tadpole density and visit as explanatory variable. The responses measured repeatedly as continuous variables were analyzed using a linear mixed effects model (lme) including the response of the trait as dependent variable and agent and tadpole density as explanatory variables and we included mesocosm as grouping (random) variable. Responses measured as categorical variables (plankton density and turbidity of water) were transformed into binomial data and analyzed using a generalized linear mixed model (glmer) with a binomial error distribution including the binomial response as dependent variable, antifungal agent, tadpole density and

visit as explanatory variable and we included mesocosm as grouping (random) variable. We ran the analysis separately for each measured response (Tab. 3.5).

2) Long-term effects on ecosystem structure: Responses on structural metrics of the ecosystem were grouped into "plankton" (consisting of 6 different variables) and "invertebrates and plants" (consisting of 4 variables). We analyzed effects of the antifungal agents and tadpole density on each category separately using MANOVA including the category as dependent variable and agent and tadpole density as explanatory variables. To analyze effects of agents and tadpole density on responses of each measured trait we fitted a linear model (lm) where we included the measured response of each trait as dependent variable and agent and tadpole density as explanatory variable (Tab. 3.6).

3) Effects on ecosystem function: Responses on functional metrics of the ecosystem were grouped into "litter breakdown" (3 variables). We analyzed effects of agent and tadpole density on litter breakdown using MANOVA including the responses in the group as dependent variable and agent and tadpole density as explanatory variables. We analyzed effects of agents and tadpole density on each single response of litter breakdown and reproduction using a linear model (lm) where we included the response as dependent variable and agent and tadpole density as explanatory variable (Tab. 3.7). In total we tested two hypotheses on how the agents act on Bd infection, six hypotheses on how the agents act on Bd transmission and 39 hypotheses concerning side effects. We lowered the usual alpha level of 0.05 with Bonferroni correction only for the results concerning effects of antifungal agents on Bd infection and Bd transmission. We did so because we wanted to avoid a false conclusion that a treatment might successfully treat amphibians against infection with Bd when in reality it did not. In contrast, when we tested for side effects, we wanted to be conservative and therefore kept alpha at 0.05. There, we were willing to accept some "false alarms".

We used R version 2.15.0 for all statistical analyses (RDevelopmentCoreTeam, 2012).

3.3 Results

Effects of the antifungal agents on Bd infection

Prevalence of infected *Alytes* was neither affected significantly by the antifungal agent ($p = 0.672$) nor by tadpole density ($p = 0.466$) (Fig. 3.2). After Bonferroni correction ($\alpha = 0.025$) we found a significant effect of antifungal agent on Bd loads among those infected *Alytes* that remained infected (lme, $p = 0.004$). Compared to the control group, General Tonic® reduced Bd loads of infected *Alytes* significantly (lme, estimate $\pm$ SE = -1.687 $\pm$ 0.546, $p = 0.005$), while the other agents had no significant effect on Bd loads of

infected *Alytes* (lme, $p_{\text{PIP Pond Plus®}} = 0.407$, $p_{\text{Virkon Aquatic®}} = 0.318$) (Fig. 3.2). Tadpole density had no significant effect on Bd loads.

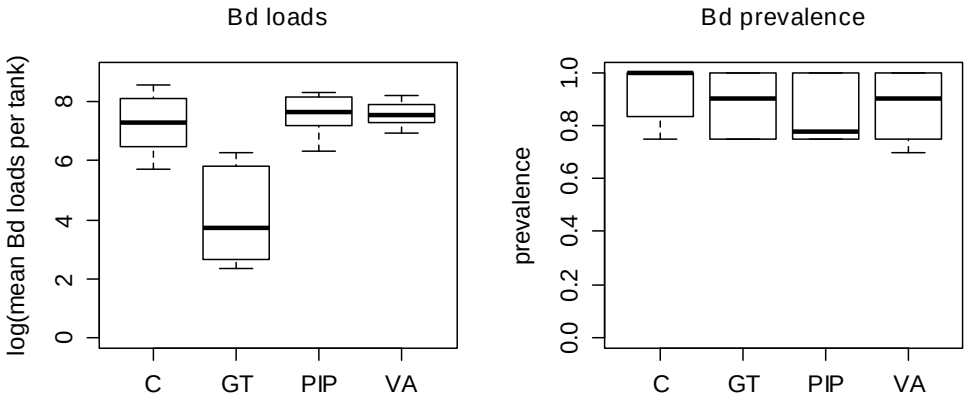

Figure 3.2: Effect of agent on the logarithm of Bd loads and on Bd prevalence of infected *Alytes*. The boxplots show infection loads of metamorphosing infected *Alytes* after four weeks of treatment with General Tonic® (GT), Virkon Aquatic® (VA), PIP Pond Plus® (PIP) or in the control group. The black line represents the median, the box represents the interquartile range containing 50% of the values and whiskers mark the 1.5 fold interquartile range.

Effects of the antifungal agents on Bd transmission

Among sentinel *Rana* only five tadpoles became infected (2 control, 1 General Tonic® , 1 Virkon Aquatic® , 1 PIP Pond Plus®); we therefore did not fit a statistical model to the sentinel *Rana* data. After Bonferroni correction ($\alpha = 0.0083$) we found a significant effect of antifungal agent on Bd prevalence in sentinel *Alytes* ($p = 0.008$). Compared to the control group prevalence of sentinel *Alytes* was neither affected significantly by General Tonic® (estimate ± SE = -0.862 ± 0.668, $p = 0.197$) nor by PIP Pond Plus® (estimate ± SE = -0.16 ± 0.607, $p = 0.792$) but Virkon Aquatic® tended to reduce prevalence in sentinel *Alytes* (estimate ± SE = -1.372 ± 0.578, $p = 0.029$). Antifungal agent showed no significant effect on prevalence in sentinel *Bufo* ($p = 0.651$) (Fig. 3.3). Tadpole density had no effect on prevalence of sentinel *Alytes* ($p = 0.103$), but we found a weak positive effect of tadpole density on prevalence of sentinel *Bufo* (estimate ± SE = 1.844 ± 0.791, $p = 0.010$) (Fig. 3.3). We found no significant effect of antifungal agent or tadpole density on Bd loads of infected sentinel *Alytes* ($p_{\text{agent}} = 0.288$, $p_{\text{tadpole density}} = 0.636$) or on Bd loads of sentinel *Bufo* ($p_{\text{agent}} = 0.142$, $p_{\text{tadpole density}} = 0.089$) (Fig. 3.3).

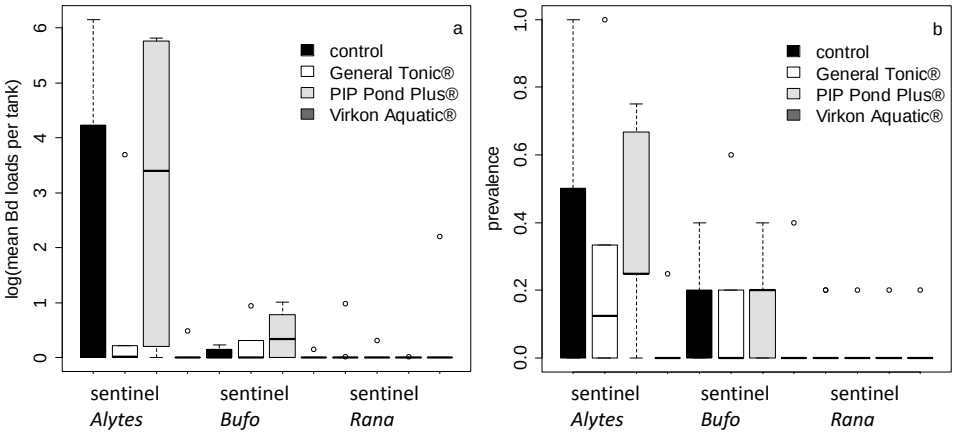

Figure 3.3: Effect of agent on transmission of Bd to sentinel tadpoles. The boxplots show infection loads of tadpoles of sentinel *Alytes*, sentinel *Rana* and sentinel *Bufo* after four weeks of treatment with different antifungal agents or in the control group. The black line represents the median, the box represents the interquartile range containing 50% of the values and whiskers mark the 1.5 fold interquartile range. Outliers are marked with circles.

Side effects of the antifungal agents on amphibians

Mass and length of infected *Alytes*, sentinel *Alytes* and sentinel *Rana*, as summarized by PC1, was neither affected by agent (lme, $p_{\text{infected } Alytes} = 0.133$, $p_{\text{seintinel } Alytes} = 0.846$; lm, $p_{\text{sentinel } Rana} = 0.523$) nor by tadpole density (lme, $p_{\text{infected } Alytes} = 0.946$, $p_{\text{sentinel } Alytes} = 0.613$; lm, $p_{\text{sentinel } Rana} = 0.923$) (Fig. 3.4). Among tadpoles of sentinel *Bufo* we found a significant negative effect of agent on PC1 (lm, $p = 0.001$) (Fig. 3.4). Compared to the control there was a significant negative effect of General Tonic® (lm, estimate ± SE = -1.854 ± 0.544, $p = 0.002$) and Virkon Aquatic® (estimate ± SE = -1.824 ± 0.544, $p = 0.003$) on PC1 scores of sentinel *Bufo*. Tadpole density showed no effect on PC1 of sentinel *Bufo* (lm, $p = 0.394$) (Fig. 3.4).

We did not detect severe histological changes in infected *Alytes*. Among the following organs we even found too few damages among all treatment groups to fit a statistical model: skin, lung, gonads, thyroid, oral mucosa and esophagus, stomach, pancreas, spleen and skeleton. Among those organs where we fit a model none of them returned significant results (logistic regression, $p_{\text{liver}} = 0.092$, $p_{\text{heart}} = 0.065$, $p_{\text{central nervous system}} = 0.182$, $p_{\text{intestine proteasis}} = 0.145$, $p_{\text{skeletal muscle}} = 0.555$). General Autolysis caused no difference among different antifungal agents (logistic regression, $p = 0.100$) however compared to the control, General Tonic® reduced general autolysis significantly (logistic regression, estimate ± SE = -2.485±1.211, $p = 0.040$), while the other agents returned no effect ($p_{\text{PIP Pond Plus®}} = 0.395$, $p_{\text{Virkon aquatic}}$

= 0.670). The tissue changes observed in the examined tissues, besides those directly related to the fungal infection, included a series of minimal to mild, more rarely moderate non-specific changes. These included hydropic degeneration and cell vacuolization in the liver, while in the kidney the collection of tubular material (amorphous material, cell debris and sloughed epithelial cells) along with occasional mineralization and cell necrosis on a single occasion were recorded. Variable amounts of eosinophilic hyaline droplets were seen in the tubules of the kidneys of the control and treatment groups except for those treated with General Tonic® , where no such structures could be seen in the examined sections. In the skeletal muscle there was multifocal minimal to mild myofiber degeneration. These changes were observed both in the controls and treatment groups. A detailed summary of the described lesions is reported in Tab. 3.2. Sporadical observations of endoparasitism were also recorded.

Table 3.2: Tissue changes observed in metamorphosing individuals of infected Alytes. HD= Hydropic degeneration; Vac.= Vacuolization; Cell R.= Cell rounding; Tub. Mat.= Tubular material including amorphous material, cell debries and sloughed tubular epithelial cells; PM= Pigmented material/macrophages; Min.= Mineralization; Nec.= Necrosis; HyD= Hyaline droplets; My.D.= Myofiber degeneration; PIP = PIP Pond Plus® ; VA= Virkon Aquatic® ; GT= General Tonic® . Full size numbers refer to the number of individuals with the specific lesions. The subscript numbers refers to the actual number of individuals examined among those available (Those excluded comprised individuals with globally or specific autolytic tissues, which could not be read or those individual for whom the specific tissue considered was not available). Superscript numbers refer to the numbers of individuals whose tissue were characterized by low-grade autolysis, which did not compromise significantly the reading and interpretation of the tissue changes.

		tissue lesions									
antifungal agent	autolysis	liver			kidney						skeletal muscle
		HD	Vac.	Cell R.	Tub.Mat.	PM	Min.	Nec.	Vac	HyD	My.D.
control	6	4_{10}	2^{1}_{10}	0_{10}	4^{3}_{10}	0_{10}	0_{10}	0_{10}	0_{10}	7^{2}_{9}	6^{1}_{10}
PIP	4	6_{10}	1_{10}	1_{10}	4^{2}_{10}	0_{10}	0_{10}	1^{1}_{10}	0_{10}	5^{1}_{9}	10^{3}_{10}
VA	5	5_{9}	5^{2}_{9}	0_{9}	3^{2}_{8}	0_{8}	0_{8}	0_{8}	1_{8}	3^{1}_{10}	8^{3}_{10}
GT	1	2_{11}	4^{1}_{11}	0_{11}	2_{11}	2_{11}	0_{11}	0_{11}	1_{11}	0_{11}	5_{11}

We found no significant effects of the factors agent and tadpole density on the activity of infected *Alytes* and sentinel *Rana* (Tab. 3.3). Activity of sentinel *Bufo* tended to be affected by the factor agent. Compared to the control group General Tonic® (lme, estimate ± SE = -10.333 ± 4.665, p

= 0.037) and Virkon Aquatic® (lme, estimate ± SE = -11.583 ± 4.665, p = 0.021) reduced the activity of sentinel *Bufo* significantly (Tab. 3.3, Fig. 3.5).

Table 3.3: Effects of antifungal agents on amphibian behavior. Analysis of deviance table of linear mixed effects model (lme) showing differences in tadpole activity among visits, among tadpole densities and among agents. Values of parameter estimates show the strength of an effect under the regime of a certain antifungal agent. Significant values are marked with * ($\alpha = 0.05$), trends with ($*$).

Species	factor or level	estimate ± SE	p-value
infected *Alytes*	visit	-0.589 ± 0.788	0.457
	tadpole density	0.104 ± 0.321	0.748
	agent		0.209
	General Tonic®	-0.625 ± 2.635	0.815
	PIP pond plus®	-3.542 ± 2.635	0.192
	Virkon Aquatic®	-5.208 ± 2.635	0.060(*)
sentinel *Rana*	visit	-0.607 ± 0.940	0.520
	tadpole density	-0.506 ± 0.425	0.245
	agent		0.618
	General Tonic®	-2.833 ± 3.483	0.424
	PIP pond plus®	-4.500 ± 3.483	0.209
	Virkon Aquatic®	-2.417 ± 3.483	0.495
sentinel *Bufo*	visit	-2.071 ± 1.428	0.150
	tadpole density	0.178 ± 0.569	0.756
	agent		0.068(*)
	General Tonic®	-10.333 ± 4.665	0.037*
	PIP pond plus®	-4.917 ± 4.665	0.303
	Virkon Aquatic®	-11.583 ± 4.665	0.021*

Side effects of antifungal agents on snails

In the short run results of MANOVA revealed that snails were significantly affected by antifungal agents and by visit but not by tadpole density (Tab. 3.4). Clutch numbers did differ among visits but not among antifungal agents or tadpole density (Tab. 3.4). We found a significant effect of antifungal agent on snail protruding from shells and on snail survival (Tab. 3.4, Fig. 3.6). General Tonic® caused the snails to protrude significantly less than in the control group while the other antifungal agents had no effect on this snail behavior (Tab. 3.4). General Tonic® also reduced survival of adult snails significantly while none of the other agents showed an effect on this response (Tab. 3.4).

Table 3.4: Short- and long-term effects of agents on snails. Multivariate and univariate analyses of responses to visit, tadpole density and agent by snails. P-values for multivariate effects are indicated in bold type. Results of the univariate analysis show differences of each response among visit, tadpole density and agent and they also show contrasts between each agent and the control group. Values of parameter estimates show the strength and direction of an effect of the respective antifungal agent. Significant values ($\alpha = 0.05$) are marked with $*$.

response variable	factor or level	estimate ± SE	p-value
	MANOVA of short term responses		
	visit		**<0.001***
	tadpole density		**0.270**
	agent		**0.005***
	Univariate LME		
number snail clutches	visit	1.346 ± 0.152	<0.001*
	tadpole density	0.032 ± 0.096	0.736
	agent		0.723
	General Tonic®	-0.675 ± 0.786	0.400
	PIP pond plus®	-0.008 ± 0.786	0.992
	Virkon Aquatic®	-0.675 ± 0.786	0.400
snail protruding	visit	-0.146 ± 0.047	0.003*
	tadpole density	-0.008 ± 0.022	0.701
	agent		0.015*
	General Tonic®	-0.483 ± 0.188	0.017*
	PIP pond plus®	0.100 ± 0.188	0.600
	Virkon Aquatic®	0.225 ± 0.188	0.244
survival adult snails	visit	-0.125 ± 0.046	0.009*
	tadpole density	-0.021 ± 0.021	0.346
	agent		0.024*
	General Tonic®	-0.475 ± 0.177	0.014*
	PIP pond plus®	-0.016 ± 0.177	0.926
	Virkon Aquatic®	0.150 ± 0.177	0.407

MANOVA of long term responses			
	tadpole density		**0.118**
	agent		**0.379**
Univariate LME			
number snail clutches	tadpole density	-0.155 ± 0.158	0.336
	agent		0.561
	General Tonic®	-0.033 ± 1.292	0.980
	PIP pond plus®	0.967 ± 1.292	0.462
	Virkon Aquatic®	1.633 ± 1.292	0.219
number snail offspring	tadpole density	0.119 ± 1.033	0.909
	agent		0.007*
	General Tonic®	-5.067 ± 8.467	0.555
	PIP pond plus®	-2.067 ± 8.467	0.809
	Virkon Aquatic®	27.267 ± 8.467	0.004*
PC1 offspring snails	tadpole density		0.330
	agent		0.810
	General Tonic®	0.312 ± 0.822	0.708
	PIP pond plus®	0.809 ± 0.885	0.372
	Virkon Aquatic®	-0.092 ± 0.822	0.911
PC1 adult snails	tadpole density	-0.209 ± 0.078	0.015*
	agent		0.076
	General Tonic®	-0.187 ± 0.688	0.788
	PIP pond plus®	-0.314 ± 0.604	0.608
	Virkon Aquatic®	1.571 ± 0.639	0.024*
survival adult snails	tadpole density	0.023 ± 0.018	0.207
	agent		0.053
	General Tonic®	-0.216 ± 0.151	0.164
	PIP pond plus®	0.033 ± 0.151	0.827
	Virkon Aquatic®	0.283 ± 0.151	0.072(*)

Results of MANOVA showed that, in the long run, snails were neither affected by antifungal agents nor by tadpole density. The number of snail clutches in the long run neither differed among agents nor among tadpole density. The number of snail offspring was affected by agent, returning significantly more offspring under the regime of Virkon Aquatic® (Tab. 3.4, Fig. 3.6). Tadpole density did not affect the number of snail offspring. Mass and length of snail offspring, summarized by PC1, did neither differ among agents nor among tadpole density (Tab. 3.4). PC1 scores of adult snails were significantly reduced at high tadpole density (Tab. 3.4). Antifungal agent tended to affect PC1 scores of adult snails. Compared to the control group Virkon Aquatic® increased PC1 scores of adult snails significantly (Tab. 3.4, Fig. 3.6). Survival of adult snails was not affected by tadpole density but tended to differ among antifungal agents. Compared to the control group Virkon Aquatic® tended to increase survival of adult snails

while the other agents showed no effect on survival (Tab. 3.4).

Side effects of the antifungal agents on structure and function of the ecosystem

Short term effects on ecosystem structure Results of MANOVA revealed significant differences in responses among visits and among agents (Tab. 3.5). All measured responses were significantly different among visits except for plankton density (Tab. 3.5). We did not find an effect of the factor tadpole density on any of the measured variables (Tab. 3.5). Agent had no effect on any of the continuous variables (Tab. 3.5). Antifungal agent had a significant effect on "plankton density" and "water turbidity". General Tonic® reduced density of plankton significantly and it caused the water to be turbid (Tab. 3.5).

Long-term effects on ecosystem structure Results of MANOVA showed that the category "plankton" tended to be affected by antifungal agent (MANOVA, $p = 0.075$) but not by tadpole density (Tab. 3.6). Within the category "plankton" we found that, *Daphnia* sp. and *Alona* sp. differed significantly in number among agents but not among different tadpole densities (Tab. 3.6, Fig. 3.7). Number of *Alona* sp. increased significantly under the regime of Virkon Aquatic® compared to the control group (Tab. 3.6, Fig. 3.7). Numbers of *Daphnia* sp. differed significantly among agents (lme, $p = 0.017$). Compared to the control group numbers of *Daphnia* sp. were significantly higher in the Virkon Aquatic® treatment (Tab. 3.6). We found no effect of agent or tadpole density on numbers of *Volvox* sp., *Cypris* sp., *Cyclops* sp. and numbers of nauplius larvae (these are larvae of crustaceans e.g. of *Cypris* sp. and *Cyclops* .sp). Compared to the control group numbers of Volvox sp. tended to be higher and numbers of *Cypris* sp. were significantly higher in the Virkon Aquatic® treatment (Tab. 3.6). Results of MANOVA revealed no effect of agent or tadpole density on the category "invertebrates and plants" (Tab. 3.6). Responses of numbers of assels, mass of beetles, molting rate of damselflies and chlorophyll content of plants did not differ significantly among agents or among tadpole density (Tab. 3.6).

Table 3.6: Long-term effects of antifungal agents on ecosystem structure. Multivariate and univariate analysis of responses by "plankton" and by "invertebrates and plants" to agent and tadpole density. P-values for multivariate effects are indicated in bold type. Results of the univariate analysis show differences of each response among tadpole density and among agent and they also show contrasts between each agent and the control group. Values of parameter estimates show the strength and direction of an effect of the respective antifungal agent. Significant values ($\alpha = 0.05$) are marked with$*$, trends with $(*)$.

response variable	factor or level	estimate	p-value
		MANOVA	
Plankton	tadpole density		**0.107**
	agent		**0.075(*)**
		Univariate LME	
Alona sp.	tadpole density	-0.435 ± 0.370	0.252
	agent		0.017*
	General Tonic®	-0.433 ± 0.506	0.401
	PIP pond plus®	0.840 ± 0.506	0.110
	Virkon Aquatic®	1.316 ± 0.506	0.016*
Daphnia sp.	tadpole density	14.071 ± 12.527	0.273
	agent		0.017*
	General Tonic®	-15.700 ± 17.116	0.369
	PIP pond plus®	-3.533 ± 17.116	0.838
	Virkon Aquatic®	46.800 ± 17.116	0.012*
Volvox sp.	tadpole density	-59.50 ± 42.69	0.177
	agent		0.130
	General Tonic®	-35.67 ± 58.32	0.546
	PIP pond plus®	-8.50 ± 58.32	0.885
	Virkon Aquatic®	114.00 ± 58.32	0.063(*)

Cypris sp.	tadpole density	-1.785 ± 1.537	0.257
	agent		0.161
	General Tonic®	0.033 ± 2.100	0.987
	PIP pond plus®	2.700 ± 2.100	0.211
	Virkon Aquatic®	4.366 ± 2.100	0.049*
Cyclops sp.	tadpole density	3.643 ± 3.921	0.363
	agent		0.595
	General Tonic®	-4.700 ± 5.358	0.389
	PIP pond plus®	-6.033 ± 5.358	0.272
	Virkon Aquatic®	-6.033 ± 5.358	0.272
Nauplius larvae	tadpole density	-0.571 ± 3.652	0.848
	agent		0.877
	General Tonic®	-3.700 ± 4.990	0.466
	PIP pond plus®	-3.700 ± 4.990	0.466
	Virkon Aquatic®	1.866 ± 4.990	0.712
Invertebrates and plants	tadpole density		**0.712**
	agent		**0.344**
counts of assels	tadpole density	-6.642 ± 13.384	0.625
	agent		0.432
mass of beetles	tadpole density	0.0006 ± 0.0005	0.279
	agent		0.510
counts of damselfly molts	tadpole density	0.429 ± 0.289	0.152
	agent		0.927
chlorophyll of plants	tadpole density	1.166 ± 1.133	0.314
	agent		0.237

Effects on ecosystem function Results of a MANOVA revealed no significant effect of agent or tadpole density on the category "litter breakdown" (Tab. 3.7). The percentage of litter breakdown of beech, oak and maple neither differed among agents nor among tadpole density (Tab. 3.7).

3.4 Discussion

The experiment mimicked a natural pond ecosystem and a possible treatment of amphibians using antifungal agents against the pathogen *Batrachochytrium dendrobatidis*. The setup of the experiment was mostly oriented at the system we find in natural ponds here. The spectrum of species we introduced into the mesocosm is very common here and the timing of introduction of the species as well is geared to the natural coming and going of species in natural ponds here. Our experiment offers insight into 1) effects of antifungal treatment on Bd infection of infected *Alytes* metamorphs; 2) effects of antifungal treatment on transmission of Bd to uninfected amphibians. Importantly, we also tested whether the antifungal treatment had side effects on amphibians and on the aquatic community and ecosystem. The

three antifungal agents differed in their effect on Bd infection. We found no effects of PIP Pond Plus® and Virkon Aquatic® on Bd infection status and infection loads of infected *Alytes* tadpoles (Woodhams et al. 2012, Fig. 3.2). In contrast, we showed that General Tonic® is effective in reducing loads of infected animals but not in clearing infection. Under the semi-natural mesocosm conditions , General Tonic® reduced loads of infected metamorphs of *Alytes obstetricans* significantly to a low level (mean GE ± SE = 174.21 ± 65.40). This result is promising because Tobler and Schmidt (2010) found that surviving metamorphs of *Alytes obstetricans* had Bd loads that exceeded the level of treated individuals in this study (mean GE ± SE of surviving *Alytes* metamorphs in Tobler and Schmidt (2010): = 282 ± 1132). We thus suggest that General Tonic® can reduce Bd loads to a level that is tolerated by *Alytes obstetricans*. Because we measured Bd loads after metamorphosis, our results furthermore show that the treatment with General Tonic® during the tadpole stage is effective throughout metamorphosis even if animals leave the treatment at stage 42 and finish metamorphosis in a Bd-friendly temperature of 19-21°C.

Transmission of Bd occurred when uninfected sentinel tadpoles became infected with Bd. Transmission occurred in all treatments. We expected that if antifungal treatments decrease loads, then transmission should be reduced. Indeed, visual inspection of the results (Fig. 3.3) suggests that infection of sentinel tadpoles was lowest when tadpoles where treated with General Tonic® and Virkon Aquatic® . The statistical analysis, however, showed that differences were mostly not significant either among density of infected *Alytes* or among antifungal agent. Results suggest that Virkon Aquatic® blocked transmission from infected to uninfected *Alytes* tadpoles even though it did not affect Bd loads. It may be that Virkon Aquatic® affects free swimming zoospores. We tentatively conclude that both General Tonic® and Virkon Aquatic® appear to reduce transmission but admit that a more powerful experiment (with more replicates and larger numbers of sentinel tadpoles) would be necessary to confirm this conclusion.

Antifungal treatments did not only affect Bd infection and transmission but also had effects of tadpoles and the aquatic ecosystem. Antifungal agents affected tadpole size and behavior but had no severe pathological effects on organs and tissue. Antifungal agents had sometimes negative effects on tadpole size (Fig. 3.4). Those effects were both agent- and species-specific. We found no negative effect of antifungal agents on size of infected and sentinel *Alytes*. This result contrasts with a previous laboratory experiment where General Tonic® had a negative effect on size of *Alytes obstetricans* tadpoles (Geiger and Schmidt 2013). Taken together, these results imply that side-effects of antifungal agents can be context-dependent and may therefore be difficult to extrapolate to other systems such as natural ponds.

The tissue changes observed in the examined individuals which might be interpreted as the expression of a presumptive toxic process were gener-

ally minimal to mild, with more rare findings of moderate alterations and were present across all treatment groups and controls. The frequent autolysis that was present in several of the examined tadpoles compromised a conclusive assessment of the actual effect, if any, of the antifungals used in this study. For a more conclusive assessment concerning the actual potential toxic effects of the drugs investigated in this study a larger sample size needs to be examined along with the ultrastructural analysis of the tissues of the treated amphibians in order to determine also those changes which cannot be obviously detected under light microscopy. Behavioral responses of tadpoles were also species- and antifungal-agent specific. The ecological consequences of reduced activity in response to General Tonic® and Virkon Aquatic® are difficult to predict. Reduced activity may lead to reduced food intake but it may also reduce predation mortality (Werner and Anholt, 1993). Reduced activity may also be a response to higher food availability (Anholt and Werner, 1998).

Our results suggest that snails respond sensitively to antifungal agents. Due to their ability to accumulate large amounts of pollutants, snails are even suggested as bioindicator species (Basopo and Naik, 2012). In the present experiment snails responded in many ways to the antifungal agents. Like in the previous discussed results, General Tonic® caused effects in the short-run while Virkon Aquatic® showed only long-term effects on snails. General Tonic® provoked a survival effect and caused the snails to retreat more into their shells. The survival effect of the adult snails disappeared in the long run and a "normal" number of offspring emerged from the General Tonic® treated mesocosms. Virkon Aquatic® had no effect in the short run. In the long run however more offspring emerged which might be due to the better survival and higher physical constitution of the adult snails. Below the line, we do not recommend a treatment with General Tonic® at sites with rare and delicate mollusks while a treatment with Virkon Aquatic® might less be of a concern but requires a careful monitoring of mollusks. Our findings on side effects on other aquatic organisms are consistent with the findings on Bd infection and on snails: effects of General Tonic® disappear after few weeks while effects of Virkon Aquatic® emerge several weeks or months after the treatment. During the month when agents were added to the mesocosms General Tonic® reduced plankton density drastically and caused the water to be turbid. Insects (damselflies and beetles) were not affected by General Tonic® . Virkon Aquatic® returned no short-term effects on any of the measured aquatic organisms.

Measurements we did several weeks after the last application of the antifungal agent inferred that short-term effects of General Tonic® disappear relatively quickly and no long-term effects emerged. However, the water in the treatment with General Tonic® remained darker and more turbid than in the other treatments. In the Virkon Aquatic® treatment we found that side effects appear only after several weeks when the number of different plank-

ton species increased. These changes implicate that Virkon Aquatic® caused changes not immediately, but may change a system over the course of some weeks, while General Tonic® changes a system immediately but not sustainably. Summarizing short- and long-term effects on structural metrics we suggest that a treatment with antifungal agents affects plankton densities causing declines immediately or increase after several weeks but it may not affect insects, crustaceans or plants heavily.

Counts of individuals, measures of water turbidity, chlorophyll content and biological diversity are point-in-time measurements that reflect the actual status or condition of an ecosystem. Therefore, we also conducted functional measurements that represent also dynamics and performance of an ecosystem (Palmer and Febria, 2012). Woodward et al. (2012) suggest litter breakdown and reproduction as a measurement of ecosystem function. In our experiment, the breakdown of oak, maple, and beech leafs was not affected by General Tonic® and neither by Virkon Aquatic® .

All side effects considered we tested 39 hypotheses of which 12 returned significant results and 3 a trend. This is a high outcome of significances, however we bring in mind that we did no Bonferroni correction and we may reckon that we registered some false alarms. A trend however is obvious: General Tonic® caused declines in a variety of responses in the short run while Virkon Aquatic® boosted numbers of individuals in the long run. Consequences of either of the two effects are difficult to predict and depend much on the system where the antifungal agent is applied.

In summary, we showed that commercially available antifungal agents can reduce Bd loads in experimental mesocosms and may also reduce Bd transmission. Negative side effects on amphibians were, in our opinion, context- and species-specific but overall relatively benign. Yet, antifungal agents have a number of side effects on the ecosystem.

Our main motivation for the experiment was to test a method inspired by use of antifungal agents in agriculture that may be used to treat amphibians against Bd in natural systems. While it is possible to treat amphibians against Bd in captivity, no treatments are available for natural systems (Lubick, 2010; Woodhams et al., 2011; Garner et al., 2012). Given our results, we believe that General Tonic® might be considered a candidate antifungal agent worth additional studies to better explore its treatment potential and side-effects to make recommendations about whether it may be used in natural environments. General Tonic® does not clear infection but it reduces Bd loads. Vredenburg et al. (2010) showed that amphibian mass mortality begins when the infection intensity reaches a critical threshold of 10,000 zoospores (the threshold might be species-specific). The models of Briggs et al. (2010) and Mitchell et al. (2008) suggest that reducing Bd infection loads might help populations to persist with endemic Bd infection. Hence, reducing Bd infection loads, rather than eradicating the pathogen, may be a viable strategy to help amphibians to persist despite the presence

of Bd. Clearly, experimental mitigation in natural systems is a necessary next step. Such experiments will show whether Bd mitigation in the field is possible.

The drawback is, of course, that Bd will persist and that repeated treatments may be necessary. As it is the case with habitat management, amphibian conservation of the future may include recurrent antifungal treatments (Woodhams et al., 2011). At best, repeated treatments might eventually reduce Bd loads permanently and might lead to the eradication of the pathogen (Lubick 2010). While this may be positive from an amphibian conservationists point of view, recurrent treatment of natural systems with antifungal agents is likely to have consequences for the ecosystem. Here, we reported some negative effects on snails and the wider ecosystem. Recurrent treatment may exacerbate such effects. The crucial question is: when shall we treat a natural system? What are the costs and the benefits? While the benefit for amphibians seems clear, we know that there are costs but the costs are much harder to quantify (Fig. 3.8). In such a situation, we recommend a precautionary approach. That is, one should only use antifungal agents in natural habitats when the expected benefit is large and likely outweighs the costs. A benefit may be large if one is witnessing a catastrophic Bd-induced population decline. In stable situations, where Bd does not appear to have an immediate catastrophic effect, e.g. Tobler et al. (2012), we would not recommend the use of antifungal agents. Costs of antifungal treatments may also depend on habitat type. Antifungal treatments may be more admissible in simple habitats (such as the gorges inhabited by the Mallorcan midwife toad *Alytes* muletensis; Woodhams et al. 2011) or man-made habitats such as gravel pits (where the common midwife toad *Alytes obstetricans* is commonly found). An assessment of costs and benefits of anti-Bd treatments should also evaluate other strategies; e.g. in the long run it may be better to block disease transmission rather than treating infected individuals (Garner et al. 2012) and approaches (e.g. use of probiotic bacteria; Woodhams et al. 2011).

While it is possible to treat natural populations against pathogens, e.g. Hudson et al. (1998), there is no such treatment yet for emerging infectious diseases such as Bd. Our mesocosm experiment is an important step towards Bd mitigation but there is still much to learn before any treatment can be recommended.

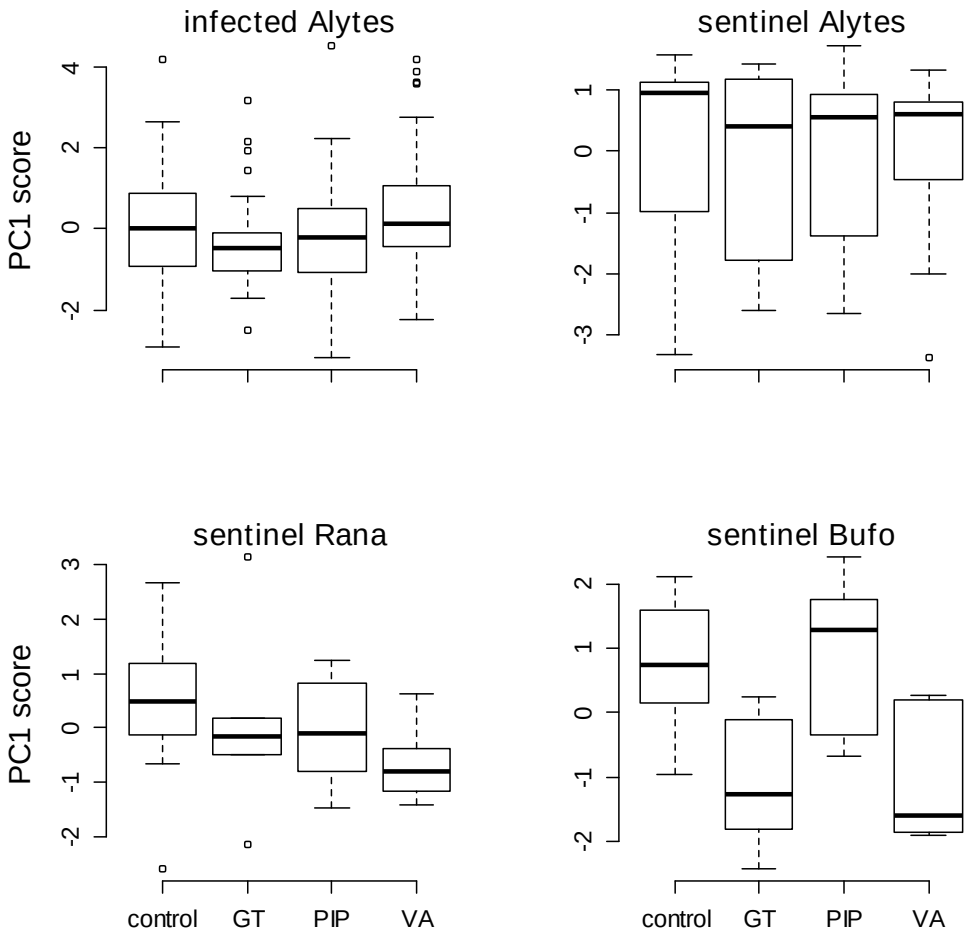

Figure 3.4: Boxplots showing effects of the antifungal agents General Tonic® (GT), PIP Pond Plus® (PIP) and Virkon Aquatic® (VA) on mass and length of metamorphosing amphibians (infected *Alytes*) and tadpoles (sentinel *Alytes*, sentinel *Bufo* and sentinel *Rana*). Mass and length is summarized by PC1 score. The black line represents the median, the box represents the interquartile range containing 50% of the values and whiskers mark the 1.5 fold interquartile range. Outliers are marked with circles.

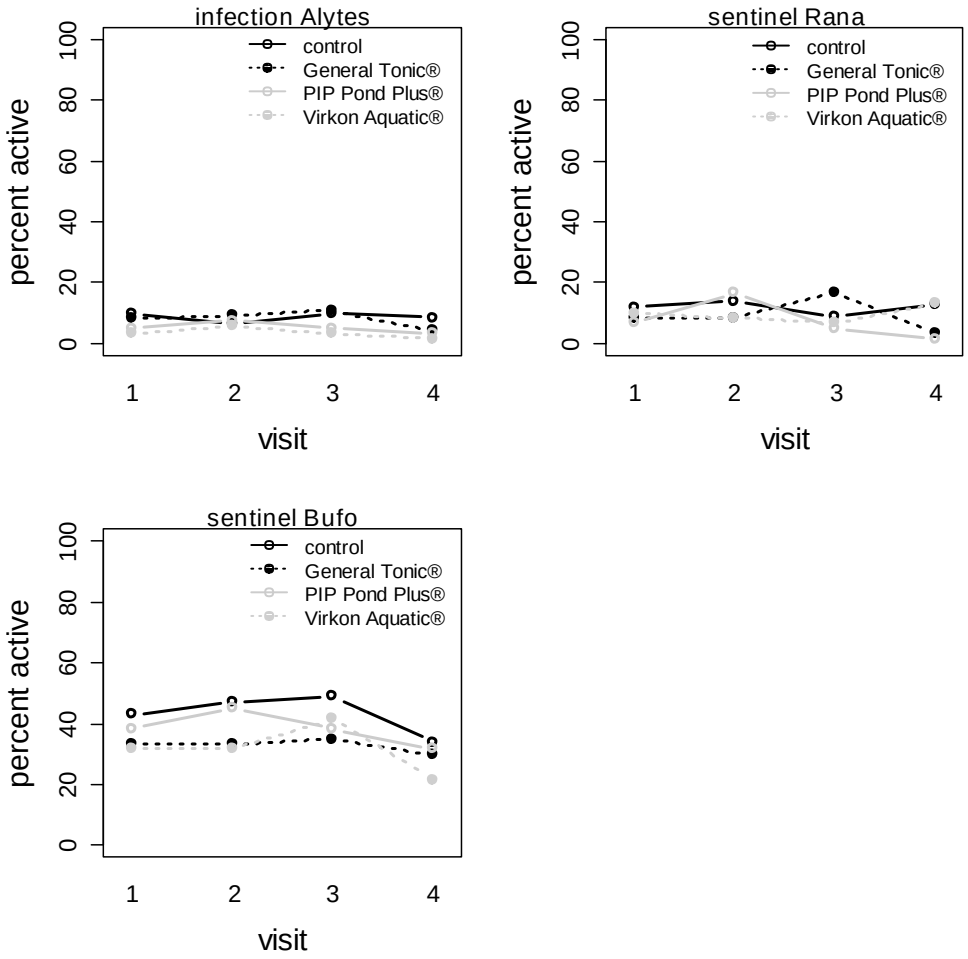

Figure 3.5: Short-term effects of agents on tadpole activity during the treatment-period. Visit 1 was right after the first treatment and visit 4 right after the last treatment. Here we calculated the percentage of tadpoles that were active (swimming or feeding) in a mesocosm.

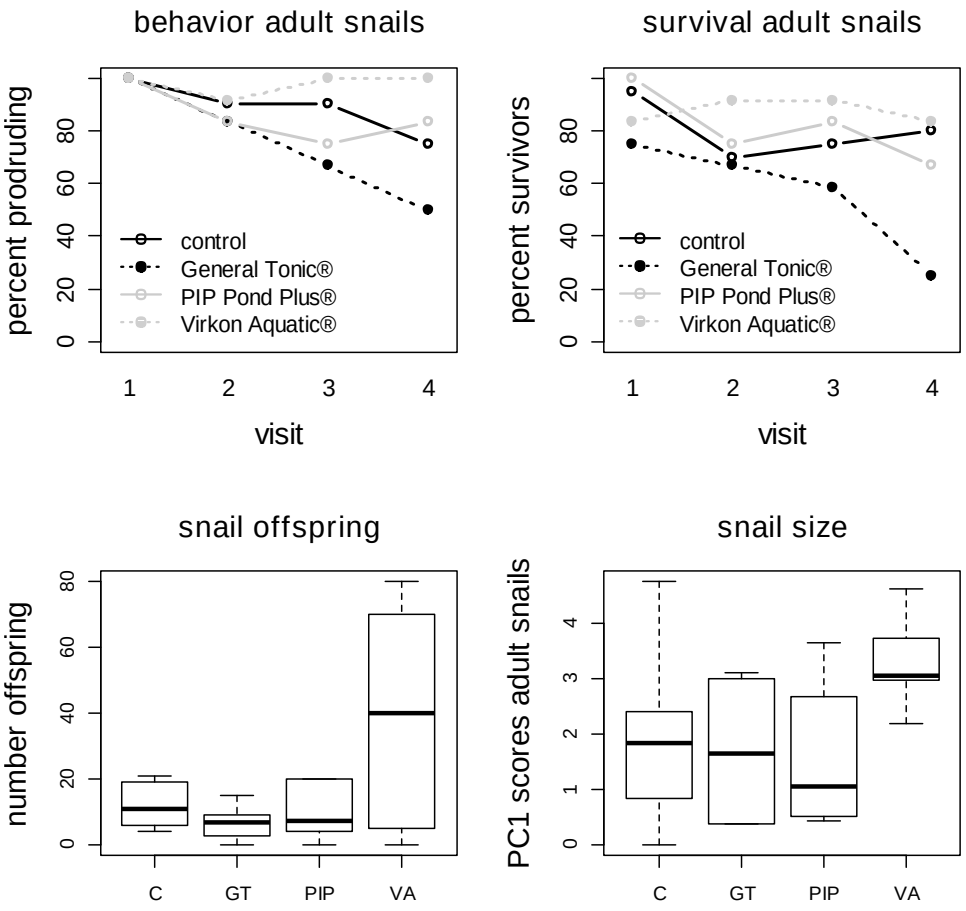

Figure 3.6: Short- and long-term side effects of antifungal agents on responses of snails. Behavior and survival of adult snails represent short-term effects measured between the first treatment (=visit 1) and the last treatment (=visit 4). Boxplots showing offspring and size of adult snails represent long-term effects measured on day 177 or 129 respectively.

Table 3.5: Short-term effects on ecosystem structure. Multivariate and univariate analysis of responses measured repeatedly during the treatment period to visit, agent and tadpole density. The response vector of the multivariate analysis is defined by the six variables of structural metrics and p-values to each factor are shown in bold type. Results of the univariate model show differences of each response among visit, tadpole density and agent and they also show contrasts between each agent and the control group. Values of parameter estimates show the strength and direction of an effect of the respective antifungal agent. Significant values ($\alpha = 0.05$) are marked with $*$.

response variable	factor or level	estimate ± SE	p-value
	MANOVA		
Short-term effects on ecosystem	visit		**<0.001**
structure	tadpole density		**0.595**
	agent		**<0.001**
	Univariate LME		
beetle activity	visit	-0.239 ± 0.066	<0.001*
	tadpole density	0.027 ± 0.027	0.326
	agent		0.559
	General Tonic®	-0.225 ± 0.219	0.314
	PIP pond plus®	0.025 ± 0.219	0.910
	Virkon Aquatic®	-0.225 ± 0.219	0.314
damselfly survival	visit	-0.079 ± 0.037	0.038*
	tadpole density	0.018 ± 0.022	0.421
	agent		0.779
	General Tonic®	-0.125 ± 0.179	0.491
	PIP pond plus®	-0.167 ± 0.179	0.360
	Virkon Aquatic®	-0.125 ± 0.179	0.491
counts of damselfly molts	visit	-0.107 ± 0.050	0.034*
	tadpole density	0.000 ± 0.018	1.000
	agent		0.536
	General Tonic®	-0.125 ± 0.152	0.419
	PIP pond plus®	0.083 ± 0.152	0.589
	Virkon Aquatic®	-0.125 ± 0.152	0.419
plankton density	visit	-0.093 ± 0.306	0.760
	tadpole density	-0.078 ± 0.116	0.498
	agent		<0.001*
	General Tonic®	-4.328 ± 0.895	<0.001*
	PIP pond plus®	0.192 ± 1.255	0.879
	Virkon Aquatic®	-0.548 ± 1.037	0.597
water turbidity	visit	0.580 ± 0.319	0.069(*)
	tadpole density	-0.112 ± 0.114	0.325
	agent		<0.001*
	General Tonic®	4.095 ± 1.137	<0.001*
	PIP pond plus®	0.537 ± 1.451	0.711
	Virkon Aquatic®	1.296 ± 1.268	0.306

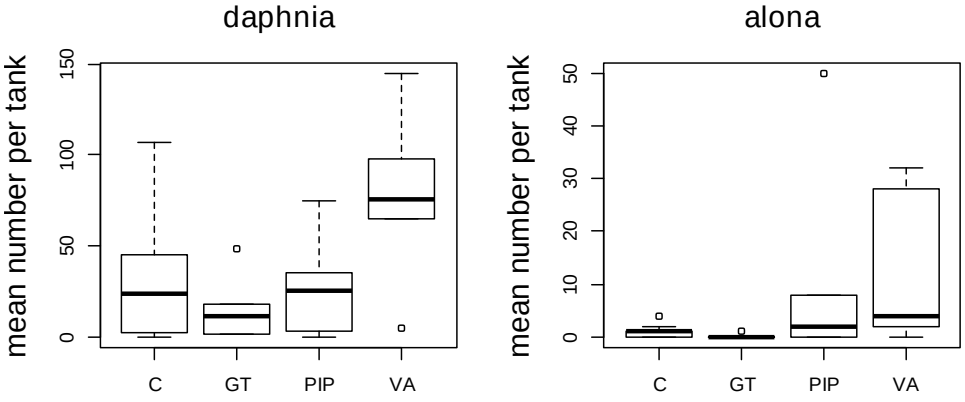

Figure 3.7: Long-term effects of the agents on ecosystem structure. Boxplots showing effects of the antifungal agents General Tonic® (GT), PIP Pond Plus® (PIP) and Virkon Aquatic® (VA) on number of *Daphnia* sp. and *Alona* sp. The black line represents the median, the box represents the interquartile range containing 50% of the values and whiskers mark the 1.5 fold interquartile range. Outliers are marked with circles.

Table 3.7: Effects of antifungal agents on ecosystem function. Multivariate and univariate analysis of responses by litter breakdown to agent and tadpole density. P-values for multivariate effects are indicated in bold type. Results of the univariate analysis show differences of each response among tadpole density and among agent and they also show contrasts between each agent and the control group. Values of parameter estimates show the strength and direction of an effect of the respective antifungal agent. Significant values ($\alpha = 0.05$) are marked with*, trends with ($*$).

response variable	factor or level	estimate	p-value
	MANOVA		
Litter breakdown	tadpole density		**0.395**
	agent		**0.725**
	Univariate LME		
% beech decomposed	tadpole density	8.107 ± 6.491	0.227
	agent		0.793
	General Tonic®	9.540 ± 9.012	0.303
	PIP pond plus®	4.585 ± 8.515	0.596
	Virkon Aquatic®	6.584 ± 9.012	0.474
% oak decomposed	tadpole density	4.825 ± 5.941	0.426
	agent		0.288
	General Tonic®	-7.016 ± 8.253	0.405
	PIP pond plus®	-12.761 ± 7.761	0.115
	Virkon Aquatic®	3.214 ± 8.253	0.701
% maple decomposed	tadpole density	-9.694 ± 5.925	0.115
	agent		0.402
	General Tonic®	-13.354 ± 8.095	0.113
	PIP pond plus®	-8.624 ± 8.095	0.298
	Virkon Aquatic®	-3.623 ± 8.095	0.659

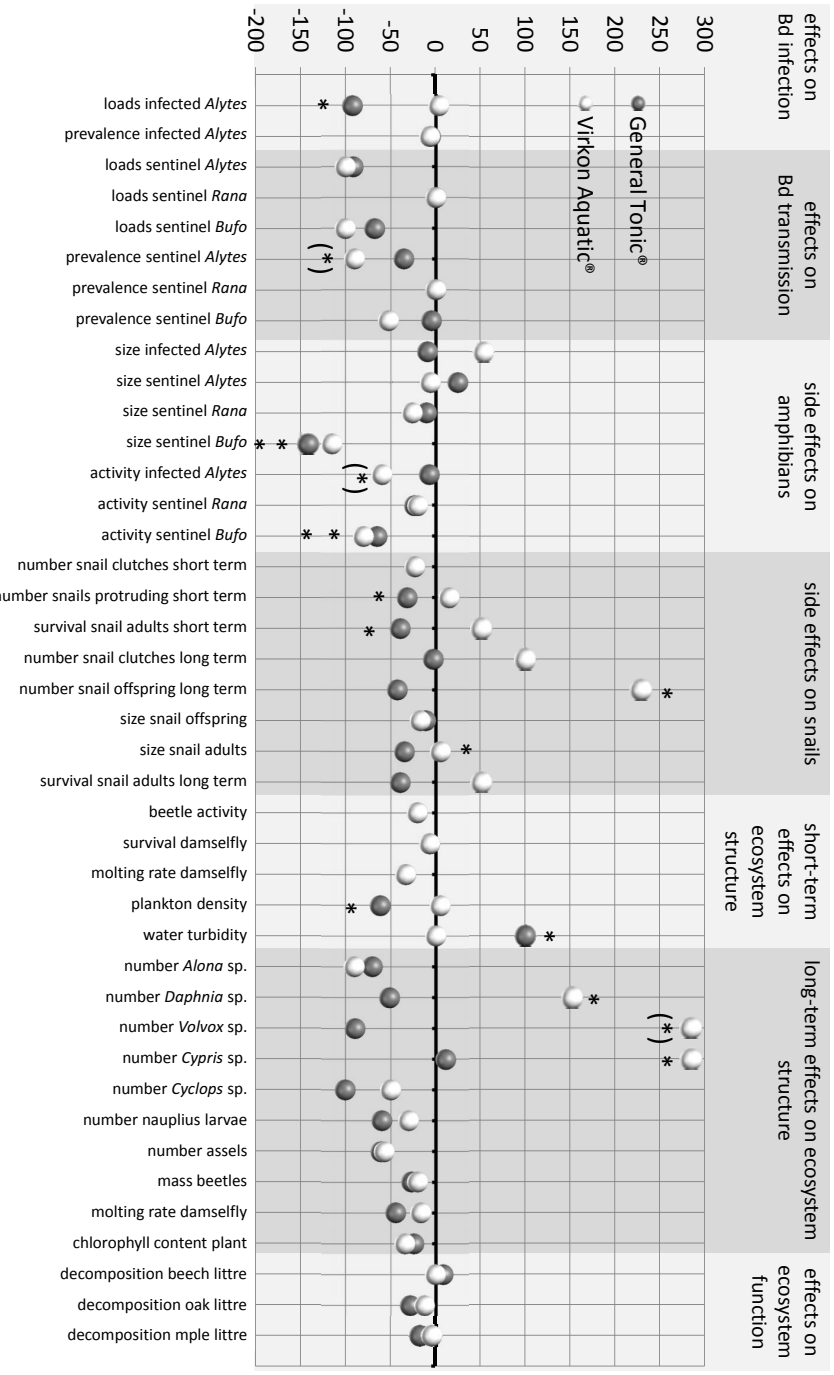

Figure 3.8: Percentaged difference of agent-induced changes in mesocosms. The mean of the traits in the control group was taken as zero. The chart shows at a glance which traits are affected by a treatment (i.e. are dislodged from the zero-line) and which traits not. Significant differences are marked with *, trends with (*).

Chapter 4

Experimental mitigation of the amphibian chytrid fungus *(Batrachochytrium dendrobatidis)* in natural ponds

Corina C. Geiger and Benedikt R. Schmidt

Abstract The disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd), is one of the most devastating emerging infectious diseases and is threatening amphibians worldwide. To halt amphibian declines, there is a need for methods that can be used to mitigate the effects of chytridiomycosis in amphibian populations. Here we report on three experiments, one in mesocosms and two in natural ponds, where we tested whether a treatment with antifungal agents during the tadpole stage reduces Bd infection in the short and in the long term. We set up experimental mesocosms and introduced infected tadpoles of *Alytes obstetricans*. After a week we treated them with Itraconazole and returned them to their original mesocosm. Upon metamorphosis we caught tadpoles out and kept them individually in the laboratory and monitored infection, survival and body size of each individual. For the second experiment we chose seven ponds for experimental antifungal treatments where infected tadpoles of *Alytes obstetricans* hibernate. In spring we caught tadpoles, treated them with Itraconazole and returned them back into their original ponds. We monitored Bd infection of treated as well as of untreated tadpoles regularly over the course of a year. We did experiment three a year later at the same sites. Experiment three was similar to the second experiment but

we applied another antifungal agent (General Tonic®) and we did treatments not only once but monthly starting in spring and ending in fall. We found that a treatment with Itraconazole in mesocosms in the tadpole stage reduced Bd loads and Bd prevalence before and after metamorphosis and reduced mortality of metamorphosed individuals. Itraconazole also reduced Bd loads and prevalence in natural ponds in the short term but not in the long term. Repeated treatments with General Tonic® reduced Bd loads in the short term but this effect was not sustained in the long run when treatments were no longer applied. Repeated treatments reduced prevalence of untreated tadpoles. This effect might be caused by the temporary removal of a subsample of infected animals during the treatment period. However we observed this effect only among young of the year tadpoles that entered the pond uninfected. Reducing tadpole density by removing individuals temporarily from a pond might thus be an effective strategy to keep prevalence on a low level, given that prevalence is low. Finally we found that a treatment in fall is not effective. These results suggest that antifungal agents can reduce infection but cannot eradicate Bd from natural environments. However, treatments are effective for a short time and thus might serve as an emergency measure in the case of a Bd-induced mass die-off.

Keywords amphibian, *Alytes obstetricans*, *Batrachochytrium dendrobatidis*, disease, mitigation, treatment, General Tonic® , Itraconazole, mesocosms, natural habitat.

4.1 Introduction

One of the most severe and increasing threats to biodiversity and wildlife are emerging infectious diseases (EIDs) (Daszak et al., 2000, 2001; Greger, 2007; Smith et al., 2009). EIDs are responsible for the decline of numerous plant and animal species resulting in an impact on the structure and function of ecological communities (Whiles et al., 2006). One of the most devastating EID is the fungal disease chytridiomycosis. It is caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd), and threatens amphibian populations around the globe (Skerratt et al., 2007). Bd is a generalist pathogen and infects of over 500 species on all continents (Fisher et al., 2009). The pathogen has apparently spread at an alarming rate over large distances and amphibian populations collapsed after emergence of the pathogen (Lips et al., 2006; Wake and Vredenburg, 2008).

Once the pathogen emerged at a place, it can persist and in some areas it is even suggested to be enzootic (Longo et al., 2010; Tobler et al., 2012). The fungus can survive up to three months in moist sterile river sand and grows on sterile feathers, dead algae and arthropod exoskeletons (Johnson and Speare, 2005). Good evidence exist that multiple asymptomatically

infected amphibian species serve as reservoir hosts (Daszak et al., 2001; Weldon et al., 2004; Picco and Collins, 2008). One important reservoir is the tadpole stage. Tadpoles can act as a within-host reservoir for the pathogen (Briggs et al., 2010) because infection in tadpoles is restricted to the mouthparts (Berger et al., 2005). Tadpoles are asymptomatic but they produce zoospores (Rollins-Smith, 1998).

In consideration of these reservoirs and diversity of hosts, a development of a mitigation strategy is a challenging issue and the complete eradication of the pathogen from an environment may be impossible (Lubick, 2010; Woodhams et al., 2011). In the present article we report on experiments in mesocosms and in natural ponds where naturally infected tadpoles were treated against Bd. We decided to focus on tadpoles because a successful treatment of tadpoles may allow amphibian populations to persist with Bd. Briggs et al. (2010) found that persistence of Bd is enhanced by the long-lived tadpole stage. Clearing infection in tadpoles or at least reducing pathogen loads shortly before metamorphosis might thus reduce mortality among metamorphosing individuals (Briggs et al., 2010; Vredenburg et al., 2010). Moreover tadpoles are often better accessible than adult individuals. Furthermore, population models lead us to expect that enhancing juvenile survival should increase population viability (Lampo and De Leo, 1998; Hels and Nachman, 2002; Conroy and Brook, 2003).

We designed three experiments in each of which we captured tadpoles in ponds and released them back into the ponds after anti-Bd treatment. We first tested the method in a mesocosm experiment under natural but controlled conditions and thereafter tested the method in natural ponds. The aim of the mesocosm experiment was to test the efficiency of an Itraconazole treatment (Garner et al., 2009a) during the tadpole stage in reduction of Bd infection, its effect on size of individuals, and also on survival of metamorphosed individuals. The aim of the two pond experiments was to test the efficiency of the antifungal agents Itraconazole and General Tonic® in reducing Bd infection (Geiger and Schmidt, 2013). The main goals of the two experiments were (i) to test whether anti-fungal treatments reduce Bd infection or infection intensity permanently and (ii) whether treatments had any side effects on treated tadpoles.

4.2 Material and Methods

Overview

This study consists of three experiments: i) A mesocosm experiment in which we tested whether Itraconazole can be used to treat tadpoles of *Alytes obstetricans* under semi-natural conditions against Bd, ii) an field experiment in which we used Itraconazole to treat tadpoles of *Alytes obstetricans* against Bd in natural ponds and iii) a field experiment in which we used

General Tonic® to treat tadpoles of *Alytes obstetricans* against Bd. We selected *Alytes obstetricans* as our model species because it is known to be susceptible to Bd (Bosch, 2001; Tobler and Schmidt, 2010; Walker et al., 2010; Böll et al., 2012).

General procedures

Antifungal agents: Itraconazole (Sporanox Oral Solution, Janssen-Cilag AG, Baar, Switzerland) was found to be an effective antifungal agent against Bd infection of tadpoles of *Alytes muletensis* and *A. obstetricans* (Garner et al., 2009a) Itraconazole cleared 100% of tadpoles of both species from Bd when they were bathed for 5 min. daily in a 1 mgL^{-1} Itraconazole solution over 7 days (Garner et al., 2009a). In *Alytes obstetricans*, no depigmentation was observed (Tobler and Schmidt, 2010). General Tonic® was developed to treat captive fish against bacterial infections, ectoparasites and for treating lesions. The active substances are ethacridinlactat, acriflavin, 9-aminoacridin * HCI * H2O and methylene blue (Tetra GmbH D 49304 Melle, Germany, www.tetra.net). In a previous laboratory and mesocosm experiment we showed that General Tonic® reduced loads and prevalence of Bd (Geiger and Schmidt 2013). DNA extractions and rt-PCR: The tips of Bd swabs were cut off, put in 60 μl of PrepMan Ultra (Applied Biosystems) and extracted applying the bead-beating protocol of Boyle et al. (2004). Extractions were afterwards diluted 1/10 and amplified following the rt-PCR protocol by (Boyle et al. 2004). Wells containing 0.01 genomic equivalents (GE) or more were scored positive. Samples were run in duplicate with negative controls and 4 dilutions of standards (100, 10, 1, 0.1 zoospore genomic equivalents, hereafter loads). If the results of the two PCR-wells were inconsistent the analysis was repeated. Values of Bd loads are corrected for the 1/10 dilution.

Mesocosm experiment

This experiment in mesocosms served as a pilot study for experiment in natural ponds.

Experimental design The experiment had a three-by-two factorial design. The first factor was population of origin. We used tadpoles from two populations (see below). The two populations were crossed with a factor "Itraconazole treatment". We kept tadpoles at a density of n=4 per mesocosms. In the control, no tadpoles were treated against Bd. In the "all" treatment, all tadpoles were treated against Bd. In the "half" treatment, 2 out of 4 tadpoles were treated against Bd. Each of the resulting 6 treatment x population combinations was replicated three times. Treatments were assigned randomly to mesocosms.

Mesocosm setup We set up 18 mesocosms in early March 2009, several weeks prior to the start of the experiment. The experimental units were rectangular fiberglass containers (1.34 m^2, 1000 L volume). We placed these containers outdoors in an open field at the University of Zurich (8.55094°E, 47.39592°N, 507 masl). The mesocosms were started with 650 L of tap water and 400 g leaf litter (*Quercus robur*, *Fagus sylvatica* and *Acer pseudoplatanus*) as substrate. Mesocosms were covered with green shade cloth to prevent colonization by invertebrates. We inoculated the mesocosms with several aliquots of pond water with concentrated plankton from a nearby wetland (Bachenbüler Allmend, 8.54295°E, 47.48309°N, 423 masl).

Stocking with amphibians All amphibians used in the experiment were from either a pond near Itingen or Zunzgen, Switzerland (Tobler and Schmidt 2010). We chose these two sites because we knew that i) *Alytes obstetricans* is abundant there, ii) tadpoles do hibernate there, iii) the site is positive for Bd and iv) the other amphibian species we needed for this experiment occur there too.

In late March 2009, we collected 146 tadpoles (72 in Itingen, 74 in Zunzgen) of *Alytes obstetricans* that had hibernated in the pond. We brought the tadpoles to the University and placed them individually in clear plastic containers. After two days we swabbed every individual over the mouthparts with a sterile rayon tipped plain swab with a plastic applicator (Copan, Brescia, Italy). Swabs were analyzed for the presence of Bd with rt-PCR. Among those tadpoles that tested positive for Bd, 72 (36 from Itingen, 36 from Zunzgen) were chosen for the experiment. Individuals were randomly assigned to one of the three treatment combinations (all, half, and control). We marked each individual with a red or orange (untreated) or rather green or pink (treated) Visible Implant Elastomer tag (VIE) (Northwest Marine Technology, Ben Nevis Loop Rd Shaw Island, Washington, USA; (Anholt et al., 1998)). To be able to recognize animals individually we put markings either on the left or on the right side of the animal. To create a realistic amphibian assemblage, we also added 20 laboratory hatched tadpoles of *Bufo bufo* and 20 of *Rana temporaria* to each mesocosm. All of them were collected in Zunzgen. Upon metamorphosis *B. bufo* and *R. temporaria* were euthanized with MS222 and stored in 98% ethanol, while the surviving *A. obstetricans* were caught out of the mesocosms and brought to the laboratory. We placed them individually in one-litre plastic containers with little tab water and with three dried beech tree (*Fagus sylvatica*) leafs (Tobler and Schmidt 2010). Containers were tilted so that both land and water were available to the toadlets. The laboratory was equipped with full spectrum sunlight lamps set for a 12 h day length. Room temperature was kept at 19 - 21°C. We fed the toadlets crickets of adequate size ad libitum three times a week. For each individual, the experiment ended 30 days after

stage 42 or upon death. After the experiment the survivors were treated with Itraconazole and released close to their original pond.

Antifungal treatments After having spent a week in the mesocosms, we caught the 72 marked and infected *Alytes obstetricans* tadpoles out of the mesocosms and brought them to the laboratory. We started the 7-day treatment by giving the "treated" tadpoles a daily bath for 5 min. in a solution of 1 mg L^{-1} Itraconazole (Garner et al. 2009, Tobler and Schmidt 2010). "untreated" tadpoles were treated the same way except that we bathed them in tap water rather than in Itraconazole solution. Tadpoles were fed *ad libitum* with fish food (Sera Spirulina Tabs, Sera GmbH, Heinsberg, Germany) all through the 7 days of their stage in the laboratory. After the treatment tadpoles were released in their original mesocosm.

Measurements We swabbed each individual at the beginning of the experiment, after the Itraconazole treatment and at Gosner (1960) stage 46 (or upon death in case that an individual died before reaching stage 46). This allowed us to test for Bd infection at the three time points "beginning", "after" and "metamorphosis". "Metamorphosis" were pooled data of the dead metamorphs (developmental stage of 45-46 and two dead individuals with stage 42) and survivors at stage 46. To assess whether antifungal treatments affects tadpole size, we recorded the body mass and body length of tadpoles at the beginning of the experiment and at stage 46. Mass was measured to the nearest 0.01g on a Scaletec Scale (Scaletec Instruments, Heiligenstadt, Germany) after blotting animals dry on a paper tissue. Body length was measured to the nearest 0.1 mm from the tip of the snout to the beginning of the tail muscle using a sliding caliper. We registered the developmental stage (Gosner, 1960) at the beginning of the experiment. We recorded the date of reaching stage 42 and the date of death or their survival until the end of the experiment (30 days after the last individual reached stage 42).

Response variables and statistical analyses of mesocosm experiment To test for effects of Itraconazole treatment and population of origin on prevalence (which was 100% at the beginning of the experiment), we used a generalized linear mixed effects model (glmer) with a binomial error distribution taking infection status of individuals (infected, not infected) as dependent variable and time, treatment, population and interaction treatment-by-population as explanatory variables (fixed effects). Mesocosm was included as grouping (random) effect (Tab. 4.1).

We used the logarithm of Bd loads to quantify infection intensity. To test for effects of Itraconazole treatment and population of origin on the logarithm of loads we used a linear mixed effects model (lme) time, treatment, population and interaction treatment-by-population as explanatory

Table 4.1: Mesocosm experiment. Effects of treatment, time, population and interaction treatment-by-population on Bd prevalence and Bd loads. P-values in bold type show differences among factors, while p-values in normal type size show contrasts in Bd prevalence and Bd loads between a certain level of a factor and the control level. Estimates and their standard errors (SE) show how strong and in which direction prevalence or loads are affected by a certain level. Significant values are marked with $*$ ($\alpha = 0.05$).

Factor	**Bd prevalence**		**Bd loads**	
level	**estimate ± SE**	**p-value**	**estimate ± SE**	**p-value**
treatment		**<0.001***		**<0.001***
half	-1.937 ± 0.703	0.006*	-2.325 ± 0.563	0.001*
all	-3.514 ± 0.770	<0.001*	-2.450 ± 0.552	0.001*
time		**<0.001***		**<0.001***
after treat.	-3.354 ± 0.602	<0.001*	-4.729 ± 0.355	<0.001*
stage 46	-3.897 ± 0.630	<0.001*	-4.681 ± 0.368	<0.001*
population		**0.964**		**0.045***
Zunzgen	-0.355 ± 0.755	0.638	-1.019 ± 0.562	0.095
treatment:population		**0.926**		**0.216**

variables (fixed effects) and mesocosm as grouping (random) effect (Tab. 4.1).

To analyze effects of the treatment on mortality we used a generalized linear mixed model (glmer) with a binomial error distribution taking mortality at the end of the experiment as dependent variable. As explanatory variables we included treatment and population as fixed effects and mesocosm as grouping (random) effect (Tab. 4.2).

To test for effects of Itraconazole treatment and population of origin on size at metamorphosis, we checked for differences in size at stage 46 among treatments and among populations (individuals that did not reach metamorphosis were excluded from this analysis). We used principal component analysis to combine mass and length of individuals into a single variable (Schmidt et al. 2012). We did one PCA for size at the beginning of the experiment and one PCA for size at stage 46. The first principal component axis (PC1) explained 97% (at the beginning of the experiment) and 85% (at stage 46) of the variance. We used the first principal component of responses at stage 46 as our measure of size at metamorphosis. We tested for an effect of treatment and population on PC1 at stage 46 using a linear mixed effects model (lme) where PC1 at stage 46 was the dependent variable and treatment, population and PC1 at the beginning were the explanatory variables

Table 4.2: Mesocosm experiment. Parameter estimates of treatment, population and density showing effects on mortality of tadpoles and on size of tadpoles at Gosner stage 46. P-values in bold type show differences among factors, p-values in normal type size show contrasts in Bd prevalence and Bd loads among levels. Estimates indicate strength and direction of an effect. Significant values are marked with * ($\alpha = 0.05$).

Factor	**mortality**		**PC1 stage 46**	
level	**estimate ± SE**	**p-value**	**estimate ± SE**	**p-value**
treatment		**<0.001***		**0.405**
half	-1.825 ± 0.649	0.005*	-0.252 ± 0.703	0.727
all	-4.289 ± 1.135	<0.001*	0.561 ± 0.684	0.428
population		**0.360**		**0.071**
Zunzgen	-0.562 ± 0.619	0.363	1.462 ± 0.601	0.032
PC1 at beginning	-	-	-0.234 ± 0.157	0.147

(fixed effects) and we included mesocosm as grouping (random) effect (Tab. 4.2).

Field experiment in natural ponds

We conducted two experiments in natural ponds. In the first experiment, we used Itraconazole to clear tadpoles of *Alytes obstetricans* from Bd infection whereas we used General Tonic® in the second experiment. In the first experiment, we treated tadpoles against Bd only once in April 2010 while in the second experiment we treated tadpoles against Bd once a month from May to September 2011. The dynamics of Bd infection were then monitored until the following spring.

Throughout both experiments we followed standard biosafety protocols to avoid spreading Bd or other pathogens (Schmidt et al., 2009b).

Experimental design Both experiments had a two-by-one factorial design. The two levels of the factor treatment (treatment and control) were randomly assigned to 7 ponds. This assignment was not changed between the Itraconazole and the General Tonic® experiment. However one site in the treatment group was dropped after the first experiment because of a lack of tadpoles. Thus in the Itraconazole experiment the level "treatment" was replicated 4 times and "control" was replicated 3 times while in the General Tonic® experiment we had 3 replicates in the level "treatment" and 3

replicates in the level "control".

Study sites The two experiments in natural ponds were carried out in the canton Lucerne in central Switzerland. This is an area where Bd does not appear to negatively affect populations despite high prevalence (Tobler et al. 2012). We selected 7 ponds located at 5 different sites: Sagerhüsli upper pond (hereafter SagO), Sagerhüsli lower pond (SagU), Räschenhaus (Rae) (only in the Itraconazole experiment), Hergiswald (Her), Hinter Rohren (HiR), Schauensee upper pond (SchO), Schauensee lower pond (SchU). The selection of the ponds was based on criteria that are exclusively related to the feasibility of the experiment: i) the sites sustain large numbers of *Alytes obstetricans* tadpoles, ii) tadpoles of *Alytes obstetricans* hibernate in the pond, iii) amphibians are positive for Bd and iv) annual counts of the number of calling males are available (Tobler et al. 2012, A. Borgula, personal communication) for an eventual evaluation of long-term effects of the treatments on population size.

The first site consists of the two ponds SagO (8.01641°E, 47.22083°N, 689 masl) and SagU (8.01740°E, 47.2210°, 672 masl). Both ponds are man-made ponds that are at a distance of about 70 m on a SE-exposed grassland. A spring supplies the water for SagO, from which it flows down to SagU. SagO is approximately 12 m^2 in size and 0.7 m in depth and is little vegetated. SagU is about 30 m^2 in size and 1.4 m in depth. One half of it is densely vegetated while the other half is nearly not vegetated. Rae (8.19382°E, 47.0152°N, 818 masl), is a former quarry in a W-exposed topographic depression. The pond is about 8 m^2 in size and 1.5 m deep, it is surrounded by reed and the ground is densely vegetated. Her (8.01740°E, 47.22101°N, 798 masl) is a static fire tank which is 15 m^2 in size and 2.5m in depth. The tank is located on a E-exposed meadow and sparely vegetated because it gets cleaned out regularly. HiR (8.18041°E, 47.00922°N, 773 masl) is a totally natural site on a W-exposed slightly afforested hillside with an aggregation of several tiny water bodies and one big pond which we included in the experiment. The pond is approximately 30 m^2 in size and 0.5 m deep. One half is in the forest where the ground is densely vegetated, the other half is in a swamp with little vegetation on the ground. SchO (8.28156°E, 47.0274°N, 570 masl) and SchU (8.28208°E, 47.0275°N, 558 masl) are two man-made ponds located 30 m from each other on a SE-exposed hillside. A fountain ensures the water supply for SchO from where it flows down to SchU. SchO is about 6 m^2 in size and about 0.5 m in depth. It is located in a concrete basin under a birch tree (*Betula pendula*) which causes the pond to have a layer of leaf litter that is cleaned out regularly by the pond owner. SchU is approximately 10 m^2 in size, 0.8 m deep and densely vegetated. SchU as well is cleaned out regularly by the pond owner.

Antifungal treatment in Itraconazole experiment We started the Itraconazole experiment in early April 2010. At the ponds where tadpoles were treated against Bd, we captured tadpoles using dipnets. A sample of n=30 was swabbed over the mouthparts with a sterile rayon tipped plain swab (Copan, Brescia, Italy) to test for Bd. All tadpoles were placed in PET bottles that were filled up to two-thirds with pond water and we transported to the laboratory at the University of Zurich. At the university, we placed tadpoles in groups of 30 in 100 L tanks filled with aged tap water. After 2 days we started the 7-day Itraconazole treatment by giving the tadpoles a daily bath for 5 minutes in a solution of 1 mg L-1 Itraconazole (Garner et al. 2009). To increase efficiency, tadpoles were treated in groups of 15 animals in tubs of 6 L volume that contained 4 L of the antifungal solution. After the 5 minute treatment tadpoles were placed back in a clean and disinfected 100 L tank that was filled up with 80 L of aged tap water. Tadpoles were fed ad libitum with fish food all through the 10 days of their stay in the laboratory. 1 day after the last treatment we swabbed 10 randomly chosen individuals to control for the success of the treatment. We marked each individual with a red VIE tag. Thereafter, tadpoles were placed for transport in clean and disinfected PET bottles that were filled up to two-thirds with tab water. All tadpoles were released at the pond of capture. These treated and marked tadpoles are hereafter called "treated tadpoles". Tadpoles from the same ponds that were not treated against Bd are called "untreated tadpoles". At the control ponds, we caught 30 individuals, swabbed and released them right after the swabbing (hereafter "control pond tadpoles"). We applied the Itraconazole treatment only once in April on tadpoles that hibernated in the ponds. This treated generation of tadpoles left the pond in summer. Consequently we do not have data of treated tadpoles for the Itraconazole treatment across years.

In order to monitor the dynamics of Bd infection after the treatment we visited each pond repeatedly (May 2010, June 2010, July 2010, September 2010, January 2011 and April 2011) and swabbed at the treated ponds 30 treated and 30 untreated tadpoles and at the control ponds 30 control tadpoles. In case that not 30 animals were available, we swabbed as many as possible. The numbers of tadpoles that were captured at each occasion is given in Tab. 4.3.

Antifungal treatment in the General Tonic® experiment We started with the General Tonic® experiment in early May 2011. At the ponds where tadpoles were treated against Bd, we captured tadpoles using dipnets. A sample of n=30 was swabbed to test for Bd. Thereafter we marked all tadpoles with pink VIE tags and placed them in groups of 50 in 100 L tanks that were filled with 50 L of tap water. Tanks were placed close to the pond and covered with green shade cloth. Tadpoles were treated against Bd using

General Tonic® in the tanks (see Geiger and Schmidt 2013). The antifungal treatment lasted eight days. During the first two days of treatment we kept concentration of General Tonic® at 1.25 ml L^{-1} . All following treatments were done with a General Tonic® concentration of 0.6 ml L^{-1} , because 13 tadpoles died during the first two days (10 in SagO and 3 in Herg). After 4 days in the tanks with General Tonic® we changed the water of all tanks and refilled them with fresh solution of 0.6 ml L^{-1} General Tonic® . 4 days later tadpoles were released into their original pond. At the control ponds we caught 30 individuals out and released them after swabbing. This procedure of treating, marking and swabbing was done in May, June, July and September 2011. At every occasion, we used a different color for the VIE tag marking. In August 2011, October 2011, January 2012 and April 2012 we monitored Bd infection of the tadpoles by swabbing 30 treated tadpoles and 30 untreated tadpoles in the treatment ponds and 30 control tadpoles in the control ponds. In case that not 30 animals were available, we swabbed as many as possible.

Measures of habitat characteristics We measured different environmental variables to explain differences in prevalence of Bd among different ponds. All ponds were equipped with one HOBO H8 temperature data logger (Onset Computer Corporation, Bourne MA, USA) that recorded water temperature 6 times a day between May 2011 and April 2012. The sensors of the loggers were placed at the deepest spot in the pond where tadpoles were found during winter 2010. Salinity, pH and oxygen concentration of the pond water were measured on the 1st of June 2012 with a HQ30d portable Multiparameter Meter (HACH Company, Loveland, Colorado, USA). We measured each parameter at three different places in each pond, each 5 cm under the water surface and approximately 0.5 m from the pond edge. We used the mean of the three measurements for statistical analysis.

Response variables and statistical analyses of field experiments in natural ponds We tested four main responses to the experimental treatments: 1) reduction of Bd loads and prevalence in the short run and 2) in the long run, 3) side effect of the monthly General Tonic® treatment on whether an individual is metamorphosing or not and 4) effect of ecological factors and experimental treatment on Bd loads and Bd prevalence.

We took June as reference month when we calculated contrasts of Bd infection among treatments because this month was available in all data sets.

1) Analysis of treatment efficiency within season in the short run: To test for the effects of experimental antifungal treatments on prevalence, we used a generalized linear mixed model (glmer) with a binomial error distribution including infection status as dependent variable and treatment, month and

interaction treatment-by-month as explanatory variables. In the General Tonic® experiment mean temperature per month was also included as an explanatory variable. Pond was included as grouping (random) variable. We used this model to compare three different results of the experiment. First, we compared "treated tadpoles" and "control pond tadpoles". Second, we compared "untreated tadpoles" and "control pond tadpoles". Third we compared "treated tadpoles" and "untreated tadpoles". The third comparison is a comparison within those ponds where some, but not all, tadpoles were treated against Bd. We ran all three analyses for the short-term time periods May to July 2010 (treated and control pond tadpoles in the Itraconazole experiment), April to September 2010 (untreated and control pond tadpoles in the Itraconazole experiment), June to October 2011 (treated and control pond tadpoles in the General Tonic® experiment) and May to September 2011 (untreated and control pond tadpoles in the General Tonic® experiment) (Tab. 4.4).

Log-transformed Bd loads of infected individuals were analysed in the same way. For the General Tonic® experiment, we included monthly mean temperature as explanatory variable.

2) Analysis of treatment efficiency across years: To analyze efficiency of agents in reducing prevalence a year after the treatment we used a generalized linear mixed model (glmer) with a binomial error distribution including infection status in April of the year following the treatment as dependent variable and treatment as explanatory variable. Pond was included as grouping (random) variable (Tab. 4.4). We followed the same approach as in the analysis within seasons comparing three different results of the experiment (first comparing "treated tadpoles" and "control pond tadpoles", second "untreated tadpoles" and "control pond tadpoles" and third comparing "treated tadpoles" and "untreated tadpoles").

Log-transformed Bd loads of infected individuals were analysed in the same way.

3) Effect of ecological factors on Bd loads and Bd prevalence: We used a model selection approach (Burnham and Anderson, 2001) to identify whether stage, salinity, pH, oxygen concentration of the pond water (O2) and mean temperature per month explained differences in Bd loads and Bd prevalence. We ranked models within 2 ΔAICc units of the best model based on Akaike's information criterion for small sample sizes (AICc) (Burnham and Anderson 2001). Because there was model selection uncertainty, we used model averaging methods to calculate parameter estimates. (Burnham and Anderson, 2002).

All statistical analyses were done in R version 2.15.0 (RDevelopmentCoreTeam, 2012).

4.3 Results

Mesocosm experiment

One animal died during the treatment with Itraconazole. In the control 4 tadpoles cleared infections, in the half treatment 3 tadpoles cleared infection and 5 got re-infected. In the all treatment none of the tadpoles got re-infected.

Infection status was significantly affected by antifungal treatment (Tab. 4.1, Fig. 4.1). Comparison of each treatment level with the control group showed significant effects of the half as well as of the all treatment on prevalence (Tab. 4.1, Fig. 4.1). Prevalence declined significantly through the course of the experiment (Fig. 4.1, Tab. 4.1). We found no significant effect of population on infection status (Tab. 4.1). The interaction antifungal treatment-by-population was not significant (Tab. 4.1).

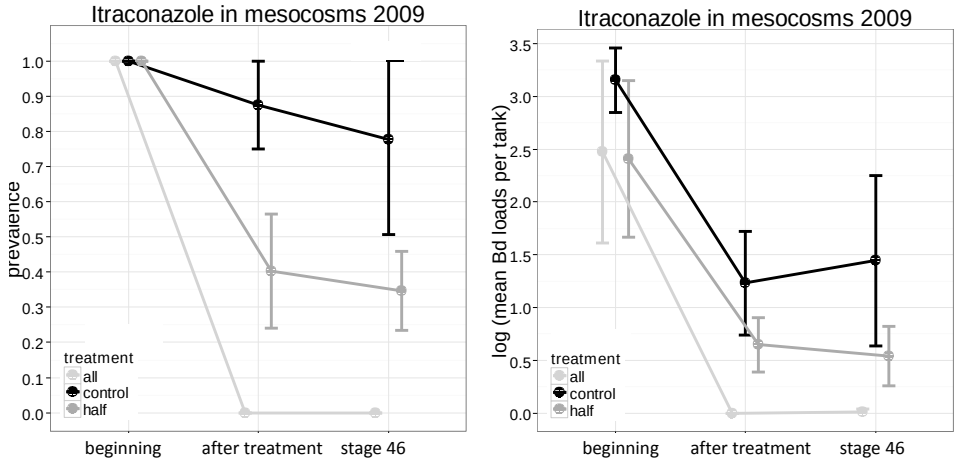

Figure 4.1: Mesocosm experiment: Effect of Itraconazole treatment on Bd prevalence and on the logarithm of Bd loads at the beginning of the experiment, after the treatment and at Gosner stage 46. Error bars represent the standard error of the mean per mesocosm.

Bd loads were significantly different among treatment groups (Tab. 4.1, Fig. 4.1). Loads differed significantly among all time periods and among populations (Tab. 4.1). Comparison of each treatment level with the control group showed significant effects of the half as well as of the all treatment on Bd loads (Tab. 4.1, Fig. 4.1). Through the course of the experiment Bd loads declined significantly (Fig. 4.1, Tab. 4.1). We found a significant effect of population on Bd loads (Tab. 4.1). The interaction antifungal treatment-by-population was not significant (Tab. 4.1).

Mortality until 30 days after stage 42 was significantly affected by treatment. Both the half treatment and the all treatment reduced mortality of

tadpoles significantly in comparison with the control (Tab. 4.2, Fig. 4.2). Population of origin had no effect on mortality (Tab. 4.2).

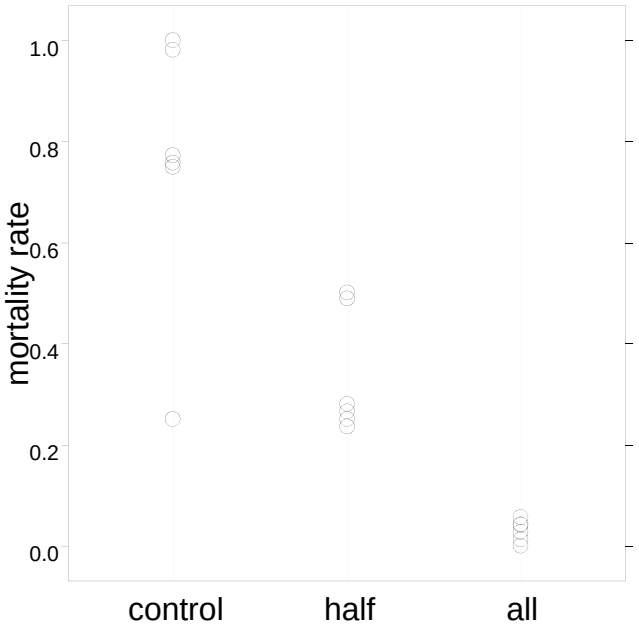

Figure 4.2: Mesocosm experiment: Effect of the different itraconazole treatments on mortality of *Alytes obstetricans*. The black line represents the median, the box represents the interquartile range containing 50% of the values. Outliers are marked with circles.

Size at metamorphosis (PC1) was neither affected by the antifungal treatment nor by population of origin and size at the start of the experiment had no significant effect either (Tab. 4.2, Fig. 4.3).

Field Experiment in natural ponds

In the course of the Itraconazole experiment in April 2010 we caught, treated and marked 580 tadpoles in the four treatment ponds (Herg: 370, SagO: 79, SagU: 51 and Rae: 80). Three tadpoles died during the Itraconazole treatment. After treatment, all tadpoles that were tested for Bd had cleared the Bd infection. The number of marked (=treated) and unmarked (=untreated) animals during the capture occasions in May, are listed in Tab. 4.3.

Numbers of treated, untreated and control tadpoles that were captured, treated and released during the General Tonic® experiment are listed in Tab. 4.3.

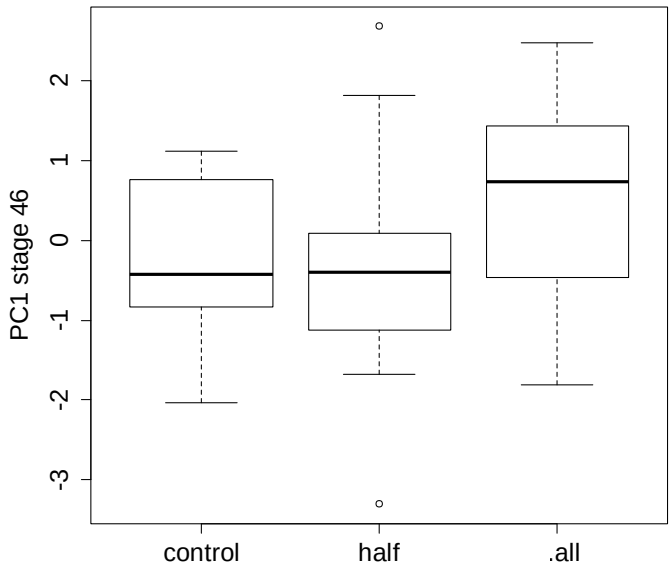

Figure 4.3: Mesocosm experiment: Effect of treatment on size of *Alytes obstetricans* at stage 46. The black line represents the median, the box represents the interquartile range containing 50% of the values and whiskers mark the 1.5 fold interquartile range. Outliers are marked with circles.

1) Analysis of treatment efficiency within season: The Itraconazole treatment caused no significant difference in Bd loads or prevalence between treated and control tadpoles (Fig. 4.4, Tab. 4.4). Bd prevalence and Bd loads of treated and control tadpoles varied significantly among months (Fig. 4.4, Tab. 4.4). The interaction between antifungal treatment and month significantly affected both prevalence and loads (Fig. 4.4, Tab. 4.4).

Comparisons of Bd loads and prevalence between untreated and control tadpoles in the Itraconazole experiment showed no difference between these two groups. Both prevalence and loads varied among months but there was no interaction between month and group (i.e., untreated vs. control tadpoles) (Fig. 4.4, Tab. 4.4).

Treated and untreated tadpoles had significantly different Bd prevalence and Bd loads. Both prevalence and loads varied among months and there was an interaction between month and group (Fig. 4.4, Tab. 4.4).

We compared Bd loads and prevalence of treated tadpoles with control tadpoles in the General Tonic® experiment. We found no significant effect of the treatment on Bd loads and Bd prevalence. Both temperature and month had a significant effect on Bd loads and on prevalence (Fig. 4.4, Tab. 4.4). The interaction between antifungal treatment and month significantly affected loads but prevalence was not affected (Fig. 4.4, Tab. 4.4).

The General Tonic® treatment did not cause a statistically significant

difference in prevalence between untreated and control tadpoles. The model returned significant effects of temperature on Bd loads and on prevalence of untreated and control tadpoles (Fig. 4.4, Tab. 4.4). Prevalence and loads were significantly different among (Fig. 4.4, Tab. 4.4). The interaction between antifungal treatment and month significantly affected both prevalence and loads (Fig. 4.4, Tab. 4.4).

Comparisons of Bd loads and prevalence between treated and untreated tadpoles in the General Tonic® experiment showed no difference between these two groups. Both temperature and month had a significant effect on Bd loads and on prevalence (Fig. 4.4, Tab. 4.4). The interaction between antifungal treatment and month significantly affected both prevalence and loads (Fig. 4.4, Tab. 4.4).

2) Analysis of treatment efficiency across years: We compared Bd loads and prevalence among untreated and control tadpoles (all treated tadpoles had metamorphosed and therefore left the pond) to test whether the Itraconazole antifungal treatment had a persistent effect one year after the treatment. We found that neither prevalence nor loads differed between these two groups of tadpoles (Fig. 4.4, Tab. 4.4).

We compared Bd loads and prevalence of treated tadpoles with control tadpoles in the General Tonic® experiment. We found no significant effect of the treatment on Bd loads and Bd prevalence (Fig. 4.4, Tab. 4.4). The General Tonic® treatment did not cause a statistically significant difference in prevalence between untreated and control tadpoles (Fig. 4.4, Tab. 4.4). Comparisons of Bd loads and prevalence between treated and untreated tadpoles in the General Tonic® experiment showed no difference between these two groups (Fig. 4.4, Tab. 4.4).

3) Effects of ecological factors on Bd loads and Bd prevalence: Differences in Bd loads were best explained by stage and by temperature with individuals in a higher developmental stage having lower Bd loads (Tab. 4.5) and individuals in higher temperature having lower Bd loads (Tab. 4.5). Differences in Bd prevalence were best explained by oxygen concentration of the pond water (O2). Individuals in ponds with low oxygen concentration had lower prevalence (Tab. 4.5).

4.4 Discussion

If there are no political, economic or social constraints, then conservation action can have a beneficial effect on threatened species (Donald et al., 2007; Hoffmann et al., 2010; Sodhi et al., 2011). Emerging diseases are a relatively new threat to biodiversity (Daszak et al., 2000; Greger, 2007; Smith et al., 2009) but there are no solutions yet how to preserve species threatened by emerging diseases (Chauvenet et al., 2011; Woodhams et al., 2011). Our aim was to develop and test a mitigation method that can be

used to treat amphibian populations against chytridiomycosis, a fungal disease which threatens amphibian diversity on many continents(Fisher et al., 2009). Our motivation to use chemical agents was inspired by agriculture where chemical agents are used to treat fungal diseases of crops (Woodhams et al., 2011). The present experiment is of the only few that tested chemical treatments in natural habitats. Similar experiments were done by Jaime Bosch in Spain on *Alytes obstetricans* (Bosch et al., 2010) or on *Alytes muletensis* in Mallorca (Jaime Bosch, personal communication). Further experiments in natural habitats were undertaken by R. A. Knapp and V. T. Vredenburg (personal communication).

Our mesocosm experiment showed that antifungal treatments in the larval stage can reduce Bd prevalence and load and that the effects persist through metamorphosis (Fig. 4.1). As a consequence, there was reduced mortality after metamorphosis (Fig. 4.2). The field experiments showed that antifungal treatments can temporarily reduce Bd prevalence and loads but have no long-term effects of pathogen prevalence and infection intensity. The dynamics of infection were not only affected by antifungal treatments but also by abiotic conditions in the pond.

The mesocosm experiment mimicked a natural pond ecosystem and amphibian community as they are commonly observed at our study sites. The treatment with Itraconazole reduced Bd prevalence and Bd loads. This effect persisted through metamorphosis and reduced post-metamorphic mortality of juveniles (Fog. 2). Post-metamorphic mortality of juveniles can be very high (Tobler and Schmidt, 2010) and may regulate amphibian population dynamics (Lampo and De Leo, 1998; Hels and Nachman, 2002; Conroy and Brook, 2003). The antifungal treatments did not affect juvenile fitness (i.e., size at metamorphosis;(Schmidt, 2012)). These results imply that treating tadpoles shortly before metamorphosis can be an efficient way to reduce chytridiomycosis-induced mortality.

The aim of the field experiments in natural ponds was to check whether the findings of the mesocosm experiment and a previous laboratory experiment (Geiger and Schmidt, 2013) can be transferred to natural ponds. Additionally, we wanted to learn whether antifungal treatments can depress Bd infection temporarily or permanently.

The efficiency of the Itraconazole treatment in reducing Bd infection was confirmed in natural ponds. We treated animals in April 2010 and measurements of Bd infection in May 2010 showed that Bd loads and Bd prevalence were still clearly reduced. However, the effect of the Itraconazole treatment disappeared after two months and Bd infection of treated tadpoles was similar to infections of the control group and untreated animals (Fig. 4.4). Bd infection of untreated animals in the pond was unaffected by the Itraconazole treatment despite that a subsample of infected tadpoles was absent for 7 days (Fig. 4.4). Briggs et al. (2010) suggested that temporarily removing tadpoles might be a strategy to reduce Bd loads. How many

animals we need to remove in order to reduce loads is difficult to predict. In the Itraconazole experiment we removed roughly 30% of the tadpoles from the ponds for the treatment (this value was estimated from the recapture data). The other question is how long tadpoles need to be out of pond. The treatment with Itraconazole took ten days, which might be too short to affect Bd infection of those tadpoles that remained in the pond. Whether percentage of removed animals, the time of the removal or a combination of both factors led to the result, is difficult to predict.

The Itraconazole field experiment showed that the effects of the antifungal treatment disappeared within months if tadpoles were treated only once. One year after the treatment we found no difference between Bd infection of tadpoles in control ponds and in ponds where tadpoles were treated. Our main motivation for the General Tonic® experiment in natural ponds was to test the effect of repeated treatments with an antifungal agent on Bd prevalence and Bd loads. The choice of the antifungal agent General Tonic® was based on results of previous experiments in laboratory (Geiger and Schmidt, 2013) and in mesocosms (chapter 3) where we found that General Tonic® reduces Bd loads significantly. The advantage of General Tonic® is the very simple application which made it possible to treat large numbers of animals repeatedly in a reasonable amount of time. In the course of the experiment with General Tonic® in mesocosms we found some side effects of the antifungal agent on non-target organisms. For this purpose we did not apply the antifungal agent directly in the pond but treated tadpoles in tanks next to the pond.

Prevalence of treated tadpoles was not reduced by the repeated General Tonic® treatments which is consistent with our findings in chapter 3. However, we found that untreated tadpoles had a lower prevalence than control tadpoles. This implies that treating a subsample of animals in the pond has an effect on those tadpoles that were not treated against Bd. It has not only an effect on prevalence but also on loads. The effect on prevalence implies that the treatment might affect transmission of the pathogen from infected to uninfected individuals. The effect on loads is difficult to interpret but might be a consequence of the effect on transmission.

Bd loads of treated and untreated tadpoles were reduced in the short term but this effect disappeared when the treatment was stopped. Shortly after the last treatment, loads of treated tadpoles leveled to the Bd loads of control tadpoles (Fig. 4.4). Results show furthermore that repeated treatments were not effective across years. Our motivation for repeated treatments was, that they might eventually reduce Bd loads permanently and might lead to the eradication of the pathogen (Lubick, 2010). Our results however returned no effect of the treatments across years. This implies that a treatment has to be applied not only during a season, but again in the next year in order to keep Bd loads in a low level.

Whether the reduction of loads with the General Tonic® treatment has

an effect on survival is difficult to predict. From our experiment with General Tonic® in mesocosms (chapter 3) we know that Bd loads of treated tadpoles remain on a low level even after metamorphosis (mean GE ± SE = 174.21 ± 65.40). This range of Bd loads is still below the Bd loads of surviving *Alytes obstetricans* metamorphs documented in Tobler and Schmidt (2010) (mean GE ± SE of surviving *Alytes obstetricans* metamorphs: = 282 ± 1132). We carefully conclude that mortality rate among metamorphosing individuals might be reduced by treating tadpoles with General Tonic® in natural ponds.

Effect of the repeated treatment was restricted to months in spring and summer. In fall Bd infection increases while temperature decreases (Fig. 4.4). This pattern was already suggested by John Bielby (personal communication) who said that high loads may be induced by low temperatures in winter. One reason for this pattern might be that cool temperature extend the time of activity of motile zoospores from 8 days (at 23°C) to 35 days (at 4°C) (Voyles et al., 2012). Zoospores encounter rates might thus be higher in low temperatures. Another reason may be that the amphibian immune system is decreased in fall, which may lead to increased susceptibility to parasites and pathogens (Raffel et al., 2006).

Taken together, a treatment with antifungal agents may reduce Bd loads and Bd prevalence of individuals in natural ponds. If loads stay on a low level over the time of metamorphosis animals might survive, as we have seen it in the Itraconazole experiment in mesocosms. In general, treatments with antifungal agents may be useful to mitigate Bd in natural ponds albeit with reasonable time and effort.

Our approach was treating tadpoles in spring shortly before metamorphosis in order to have reduced Bd infection when susceptibility of animals is highest, i.e. after metamorphosis. The mesocosm experiment showed that this approach is effective. In the course of the field experiments in natural ponds we observed that prevalence and loads were lowest in fall. A new approach might thus be to treat animals in fall in order to keep Bd infection on a low level. Another observation was that ecological factors affect Bd infection. Individuals in high temperatures have low Bd loads. This brings us to another approach which is the treatment with elevated temperature. This idea was already tested in the laboratory and is an effective way to reduce loads or even to clear individuals from Bd (Geiger et al., 2011; Woodhams et al., 2011). This idea might be worth testing in natural ponds either by treating individuals in tanks with warm water next to the pond or by creating shallow water zones in order to provide warmer water. Warmer water speeds up larval development which implies that fewer tadpoles will hibernate (Thiesmeier, 1992) and therefore population growth rate will increase (Govindarajulu et al., 2005). Speeding up larval development might additionally reduce Bd loads because analysis of Bd infection under different ecological factors showed that individuals in a higher developmental stage

have lower Bd loads.

The drawback of course is that Bd will persist and repeated treatments are necessary to keep Bd infection on a low level. In some sites repeated treatments are a possibility to reduce Bd infection i.e. at sites where tadpoles are easy accessible. In many cases tadpoles are very difficult to access and the approach might thus be difficult to apply or only with a considerable amount of time and manual effort. Yet in an emergency case like after a Bd-induced collapse of a population we have a method in hand which allows reducing Bd prevalence and Bd loads in natural habitats.

Table 4.3: Field Experiment in natural ponds: Number of *Alytes obstetricans* tadpoles that got sampled during the two experiments in natural ponds. treat = treated tadpoles, untreat = untreated tadpoles.

	treatment ponds								**control ponds**		
	SagO		**SagU**		**Herg**		**Rae**		**SchO**	**SchU**	**HiR**
	treat	untreat	treat	untreat	treat	untreat	treat	untreat	control	control	control
Itraconazole experiment 2010											
April 10	79	0	51	0	370	0	80	0	30	29	31
May 10	30	7	18	11	30	25	25	11	30	30	30
June 10	35	5	14	16	30	11	21	8	19	31	27
July 10	32	14	3	15	36	8	9	4	15	29	13
September 10	0	28	0	29	0	32	1	32	32	33	30
January 11	0	30	0	30	0	30	2	19	30	30	30
April 11	0	30	0	31	0	30	0	12	30	30	29
General Tonic® experiment 2011											
May 11	111	0	187	0	363	0	-	-	30	31	13
June 11	74	37	58	66	187	355	-	-	30	30	22
July 11	74	6	16	9	97	115	-	-	29	30	21
August 11	5	31	0	30	13	906	-	-	20	20	30
September 11	3	278	0	461	0	912	-	-	30	28	30
October 11	24	3	20	10	17	25	-	-	30	30	29
January 12	66	9	30	30	63	30	-	-	30	30	30
April 12	63	8	31	43	76	34	-	-	30	30	26

Table 4.4: Field Experiment in natural ponds. Differences in Bd prevalence and Bd loads among treatment, month and temperature within seasons. We analyzed three different data sets which included i) treated and control pond tadpoles, ii) untreated and control pond tadpoles and iii) treated and untreated tadpoles. P-values show differences among factors, Significant values are marked with* for a significance level of $\alpha = 0.05$. Itra = Itraconazole, GT = General Tonic.

factor	**treated-control**		**untreated-control**		**treated-untreated**	
	loads	prevalence	loads	prevalence	loads	prevalence
			within season			
Itraconazole	0.208	0.365	0.517	0.689	0.002*	0.039*
month	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Itra:month	<0.001*	<0.001*	0.255	0.053	<0.001*	<0.001*
General Tonic®	0.193	0.738	0.952	0.355	0.494	0.122
temp	<0.001*	0.011*	<0.001*	0.006*	<0.001*	0.042*
month	<0.001*	0.004*	<0.001*	<0.001*	<0.001*	0.009*
GT:month	<0.001*	0.654	<0.001*	<0.001*	0.597	0.970
			across years			
Itraconazole	-	-	0.134	0.232	-	-
GT	0.139	0.205	0.307	0.724	0.135	0.394

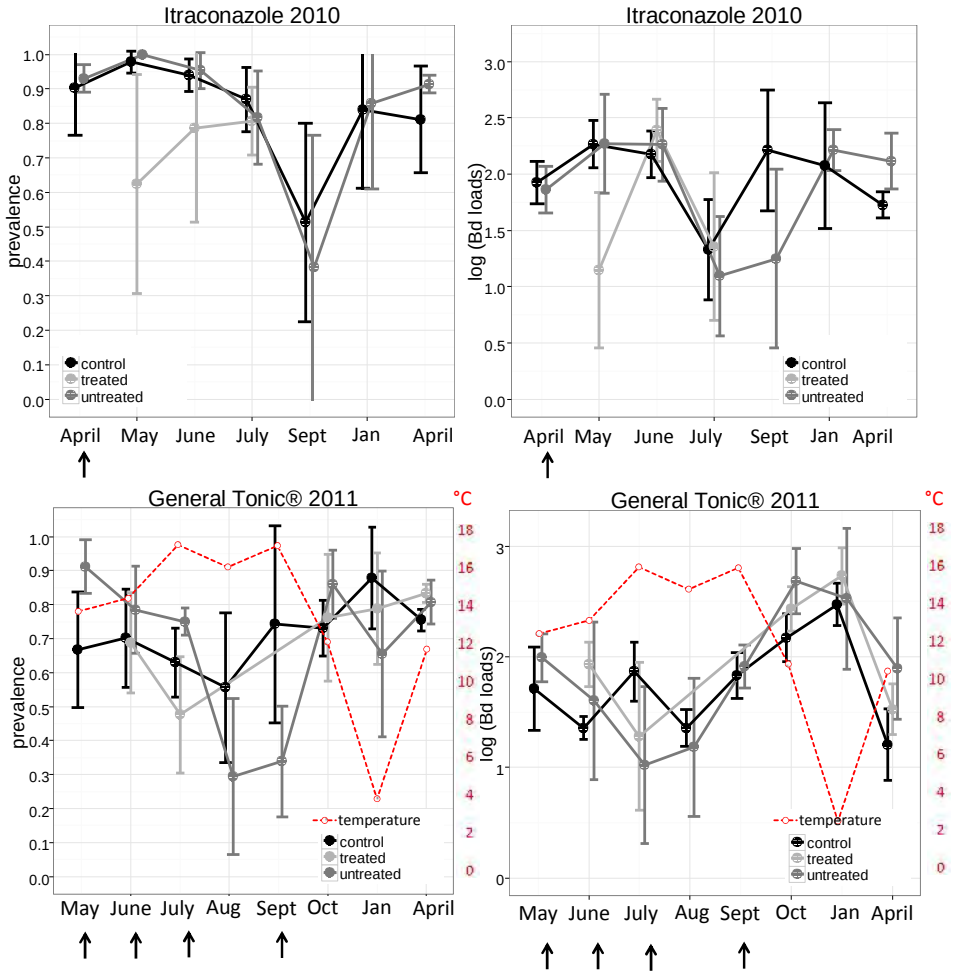

Figure 4.4: Effect of itraconazole treatment and General Tonic treatment on Bd prevalence and on the logarithm of Bd loads over the time period of one year. Error bars represent the standard error of the means per pond. Arrows indicate in which month a treatment was done. Temperatures are shown as a mean water temperature over all ponds.

Table 4.5: Effect of ecological factors on Bd loads and prevalence. Model selection results of linear mixed effects model (lme) for loads and of generalized linear models (glmer) for Bd prevalence.

					estimates ± SE				
	df	ΔAICc	weight	likelihood	O2	pH	salinity	stage	temperature	treatment
Bd loads										
stage + salinity	6	0	0.204	1.000			10.100 ± 4.281	-0.048 ± 0.012		
stage + salinity + temperature	7	0.01	0.203	0.995			8.292 ± 4.488	-0.047 ± 0.012	-0.100 ± 0.049	
stage + temperature	6	1.24	0.110	0.538				-0.046 ± 0.012	-0.122 ± 0.047	
stage + temperature + pH	7	1.4	0.102	0.497		-1.523 ± 1.109		-0.046 ± 0.012	-0.125 ± 0.047	
stage + salinity + pH	7	1.96	0.077	0.375		0.353 ± 1.380	11.040 ± 5.638	-0.048 ± 0.012		
stage + salinity + temperature + treatment	8	1.96	0.077	0.375			7.719 ± 4.917	-0.047 ± 0.012	-0.100 ± 0.050	0.105 ± 0.368
stage + salinity + treatment	7	1.97	0.076	0.373			9.656 ± 4.698	-0.048 ± 0.012		0.082 ± 0.356
stage + salinity + O2	7	1.99	0.076	0.370	0.012 ± 0.066		9.754 ± 4.698	-0.048 ± 0.012		
stage + salinity + temperature + pH	8	2.00	0.075	0.368		-0.307 ± 1.466	7.421 ± 6.067	-0.047 ± 0.012	-0.103 ± 0.050	
model averaged parameter estimates	**10**	**-**	**1.000**	**0.565**	**0.012 ± 0.067**	**-0.594 ± 1.533**	**9.162 ± 4.948**	**-0.047 ± 0.012**	**-0.109 ± 0.050**	**0.093 ± 0.363**
Bd prevalence										
temperature + O2 + stage + salinity	5	0	0.136	1.000	-0.089 ± 0.024	0.000	3.887 ± 1.790	-0.021 ± 0.012	-0.025 ± 0.014	
temperature + stage + pH + treatment	5	0.1	0.129	0.951		-1.092 ± 0.398		-0.020 ± 0.012	-0.026 ± 0.014	-0.318 ± 0.122
temperature + O2 + stage + pH	5	0.5	0.106	0.779	-0.060 ± 0.023	-0.842 ± 0.409		-0.020 ± 0.012	-0.029 ± 0.014	
temperature + pH + treatment	4	0.74	0.094	0.691		-1.066 ± 0.397			-0.023 ± 0.014	-0.314 ± 0.122
temperature + O2 + salinity	4	0.89	0.087	0.641	-0.087 ± 0.024		3.609 ± 1.780		-0.022 ± 0.014	
temperature + O2 + pH	4	1.03	0.081	0.598	-0.060 ± 0.024	-0.817 ± 0.409			-0.025 ± 0.014	
O2 + stage + salinity	4	1.26	0.072	0.533	-0.091 ± 0.024		3.932 ± 1.786	-0.018 ± 0.012		
O2 + salinity	3	1.43	0.066	0.489	-0.089 ± 0.024		3.687 ± 1.775			
pH + treatment	3	1.53	0.063	0.465		-1.013 ±0.396				-0.340 ± 0.121
stage + pH + treatment	4	1.62	0.060	0.445		-1.028 ± 0.396		-0.017 ± 0.012		-0.347 ± 0.122
temperature + O2 + stage + pH + salinity	6	1.76	0.056	0.415	-0.078 ± 0.031	-0.354 ± 0.691	2.644 ± 3.019	-0.021 ± 0.012	-0.027 ± 0.014	
temperature + O2 + stage + salinity + treatment	6	2.01	0.050	0.366	-0.093 ± 0.054		3.860 ± 1.820	-0.021 ± 0.012	-0.025 ± 0.014	0.024 ± 0.291
model averaged parameter estimates	**7**	**-**	**1.000**	**0.614**	**-0.080 ± 0.031**	**-0.920 ± 0.486**	**3.661 ± 2.017**	**-0.020 ± 0.012**	**-0.025 ± 0.014**	**-0.282 ± 0.193**

Chapter 5

Learning from failed trials

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Abstract Amphibian conservation goals depend on effective disease-treatment protocols. Desirable protocols are species, life stage, and context specific, but currently few treatment options exist for amphibians infected with the chytrid fungus *Batrachochytrium dendrobatidis* (Bd). Treatment options, at present, include antifungal drugs and heat therapy, but risks of toxicity and sideeffects make these options untenable in some cases. Here, we report on the comparison of several novel treatments with a more generally accepted antifungal treatment in experimental scientific trials to treat Bd-infected frogs including *Alytes obstetricans* tadpoles and metamorphs, *Bufo bufo* and *Limnodynastes peronii* metamorphs, and *Lithobates pipiens* and *Rana muscosa* adults. The experimental treatments included commercial antifungal products (itraconazole, mandipropamid, steriplantN, and PIP Pond Plus), antimicrobial skin peptides from the Bd-resistant *Pelophylax esculentus*, microbial treatments (Pedobacter cryoconitis), and heat therapy (35°C for 24 h). None of the new experimental treatments were considered successful in terms of improving survival; however, these results may advance future research by indicating the limits and potential of the various protocols. Caution in the use of itraconazole is warranted because of observed toxicity in metamorphic and adult frogs, even at low concentrations. Results suggest that rather than focusing on a single cure-all, diverse lines of research may provide multiple options for treating Bd infection in amphibians. Learning from failed treatments' is essential for the timely achievement of conservation goals and one of the primary aims for a publicly accessible treatment database under development.

Keywords *Alytes obstetricans*, *Batrachochytrium dendrobatidis*, Biotherapy, *Bufo bufo*, Chytridiomycosis, Disease control, *Lithobates pipiens*, Probiotic, *Rana muscosa*

5.1 Introduction

Amphibians are more threatened than any other vertebrate taxon (Stuart et al., 2004; Hoffmann et al., 2010). One significant factor contributing to population declines is an emerging infectious disease called chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (Bd) (Berger et al., 1998; Daszak et al., 2000; Skerratt et al., 2007). Chytridiomycosis causes death to amphibians both in the laboratory (Parker et al., 2002; Tobler and Schmidt, 2010) and in natural habitats (Berger et al., 1998; Bosch, 2001; Lips et al., 2006; Pounds et al., 2006). The disease is associated with mass mortality events, population declines (Vredenburg et al., 2010), the loss of amphibian biodiversity (Daszak et al., 2003; Lips et al., 2006; Smith et al., 2009), and subsequent ecosystem changes (Whiles et al., 2006). Chytridiomycosis was charactereized in the IUCN Amphibian Conservation Action Plan as "the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (Gascon et al., 2007). Hence, it is of particular importance to develop effective methods to mitigate Bd (Woodhams et al., 2011) and also to cure infected amphibians (Mendelson et al., 2006).

The urgent development of antifungal treatment protocols has resulted in several reports (Nichols and Lamiranda, 2000; Berger et al., 2010; Bowerman et al., 2010; Pessier and Mendelson, 2010; Martel et al., 2011; Tamukai et al., 2011). Successful trials have been documented in peer-reviewed journals. These reports include treatment with elevated temperature (Woodhams et al., 2003; Chatfield and Richards-Zawacki, 2011; Geiger et al., 2011), treatment with salt (White, 2006), and also treatments with different antifungals (Hadfield and Whitaker, 2005; Garner et al., 2009a; Bowerman et al., 2010; Martel et al., 2011). Unfortunately, information about unsuccessful trials is scarce (Berger et al., 2009). Anecdotal reports of both successful and unsuccessful treatments used in the pet trade, zoos, and for captive assurance colonies are becoming more numerous. These unpublished reports may help to guide future clinical studies. To be efficient in developing successful mitigation methods against Bd, intuitive yet unsuccessful trials should not be needlessly repeated. Thus, documentation of failed trials is important for future research planning.

Here, we report on 5 experiments that were considered unsuccessful treatments in terms of improving amphibian survival, and we also outline what can be learned from these unsuccessful treatment attempts. First, we examine treatments of newly metamorphosed common toads *Bufo bufo*.

This species is common, yet susceptible to chytridiomycosis (Bosch and Rincon, 2008; Fisher et al., 2009; Garner et al., 2009b, 2010) and, therefore, a good model species for development of treatment protocols. Since the species does not appear to produce conventional cationic antimicrobial skin peptides (Roseghini et al., 1989), we chose to examine the effects of adding anti-Bd skin peptides from a disease- resistant species. If the added antimicrobial peptides reduce the pathogen load, the adaptive immunesystem may be better able to clear infections. Second, adult mountain yellow-legged frogs *Rana muscosa* were treated with either itraconazole or the anti-Bd bacterium *Pedobacter cryoconitis*. Third, itraconazole and heat treatments were tested on infected adult northern leopard frogs *Lithobates pipiens*. Successful treatment of this infection-tolerant reservoir host (Woodhams et al., 2008) could be implemented by commercial suppliers to reduce the spread of Bd. Metamorphosing striped marsh frogs *Limnodynastes peronii* and midwife toads *Alytes obstetricans* show a typical pattern of chytridiomycosis development at metamorphosis (Marantelli et al., 2004; Tobler and Schmidt, 2010), and treatments with several antifungal compounds were tested. Treatment at the tadpole stage may reduce the incidence of disease in later stages of host development. These experiments test a broad range of potential treatment protocols across a variety of life stages and contexts important for mitigating the emerging amphibian disease chytridiomycosis.

5.2 Material and Methods

Treating newly metamorphosed *Bufo bufo* with antimicrobial peptides

Animal husbandry Four amplexing pairs of common toads *Bufo bufo* were collected at the pond on the Färberwiesli, near Schaffhausen, Switzerland (47° 42' 1.19" N, 8° 35' 42.45" E) in March 2010, and kept in captivity overnight for egg collection. Tadpoles were reared in outdoor artificial ponds (0.28 m^2 plastic tubs containing 80 L of water) at the University of Zurich. Artificial ponds were disinfected before use with Virkon S (DuPont), covered with shade cloth, provided with leaves and zooplankton to establish semi-natural conditions, and irregularly supplemented with fish feed (Sera Spirulina Tabs; Sera GmbH). Upon metamorphosis, toadlets were transferred to tubs with access to land and water and fed crickets, aphids, and fruit flies. Temperature in the tubs fluctuated naturally and reached 30°C on several occasions. On July 12, 2010, 75 *B. bufo* metamorphs (mean ± SD: 0.094 ± 0.029 g) were placed in a controlled environment room kept between 17 and 18.5°C on a 14 h light:10 h dark schedule with full-spectrum lighting. Each toadlet was given a new plastic enclosure tipped to one side containing approximately 25 ml of aged tap water, and fed crickets 2 to 3 times weekly during the laboratory experiment. Relative humidity was consistently 100%

in both outdoor tubs and laboratory enclosures. Temperature and humidity were recorded with LogTag Recorders. Under these husbandry conditions, the toads were considered to be free of Bd infections at the beginning of the treatments.

Experimental design The toadlets were randomly allocated to 1 of 5 treatment regimes and placed in a randomized block design. Initial mass did not significantly differ among treatments (ANOVA: $F = 0.907$, $df = 4$, $p = 0.465$). Toadlets were exposed 1 time to either Bd (7 105 zoospores mixed from UK *Bufo bufo* isolate and Swiss *Alytes obstetricans* isolate 0739) or a sham solution of water washed from sterile plates by placing in a sterile 15 ml tube with 1 ml solution for 1 h. Peptides were rinsed from *Pelophylax esculentus* skin after norepinephrine induction (40 nmol g-1 body mass) of granular gland secretions. Peptide treatment consisted of a 2 min bath in 1 ml solution containing 400 μg ml-1 peptide mixtures collected from 15 P. esculentus and partially purified over C-18 Sep-Paks (Waters Corp.) and combined. This peptide concentration was approximately the minimal concentration needed to completely inhibit growth of Bd

(D.C. Woodhams unpubl. data). In Treatment 1, toad lets (n = 12) were unexposed controls. In Treatment 2 toadlets (n = 12) were not exposed to Bd but treated with peptides. In Treatment 3 toadlets (n = 17) were exposed to Bd. In Treatment 4 toadlets (n = 17) were exposed to Bd immediately after treatment with peptides. In Treatment 5 toadlets (n = 17) were exposed to Bd and then treated with peptides on Days 8 and 9 after exposure. Toadlets were monitored daily for clinical signs of disease and weighed 2 and 5 wk after exposure. At the end of the experiment on Day 35, the skin of toadlets was swabbed for quantitative real time PCR (qPCR) diagnostic analysis of Bd infection according to Hyatt et al. (2007). Standard statistical analyses were carried out using IBM SPSS Statistics 19 (SPSS Inc.) for this and the following experiments.

Treating adult *Rana muscosa* with itraconazole and biotherapy

Animal husbandry Forty-four adult *Rana muscosa* (mean ± SD: 8.8 ± 2.1 g) were collected from Sixty Lake Basin in the Sierra Nevada mountains of California, USA, transported by helicopter out of the park in individual plastic containers, and shipped overnight to James Madison University, Harrisonburg, Virginia, USA, arriving on July 13, 2007. These lakes were known to be heavily infected with Bd and were thought to be in imminent danger of population collapse caused by chytridiomycosis. One frog was moribund upon collection in the field and died 2 d after arrival. Upon arrival at the laboratory, each frog was swabbed on the left side only for qPCR diagnostic analysis of Bd, as above. Each frog was then rinsed in sterile artificial pond

water (Provisoli medium, Wyngaard and Chinnappa 1982) to remove debris and transient microbes, and swabbed on the right side for analysis of skin microbiota by denaturing gradient gel electrophoresis (DGGE, described in DGGE'). Next the frogs were weighed, treated, and placed individually into 2 L plastic containers containing a 5 cm plastic saucer and approximately 200 ml of artificial pond water. All containers were randomly assigned a position on metal racks in a controlled environment room set at 17°C with a 12 h light:12 h dark cycle. Containers were cleaned with 10% bleach and autoclaved twice per week. Artificial pond water was autoclaved and cooled before use. Frogs were fed crickets twice per week.

Experimental design Frogs were randomly allocated to 3 treatments: control (n = 13), biotherapy (n = 20), and itraconazole (n = 10). Analysis of variance showed no significant differences among treatments in initial mass (mean ± SD: 8.8 ± 2.1 g, F = 0.72, df = 2, p = 0.493) or Bd load (mean ± SD: 46 888 ± 92 716 zoospore equivalents, F = 0.371, df = 2, p = 0.692). Four days after arrival, control frogs were treated by placing them in 25 ml of artificial pond water for 2 h and swishing' every 30 min. Biotherapy frogs were treated similarly, except that the water contained Pedobacter cryoconitis at a concentration of approximately 1.57 108 cells ml-1. The P. cryoconitis strain used was originally isolated from a wild adult *Rana muscosa* from Sixty Lake Basin in August, 2005, and found to inhibit Bd growth in co-culture assays (Woodhams et al. 2007). A pure culture was incubated for 24 h at room temperature in 1% tryptone with continuous stirring. The culture was then centrifuged at 4500 g for 10 min at 10°C, and the supernatant was discarded. The cells were then washed by re-suspending the pellet in artificial pond water and repeating the centrifugation twice. A third group of frogs was treated for 11 d with itraconazole by placing each frog in a plastic container for 5 min with 25 ml artificial pond water containing 250 ul of Sporonox oral solution at 10 mg ml-1 (final concentration: 100 mg L^{-1} ; Nichols et al. 2000). All frogs were weighed and swabbed for Bd each week, and microbiota swabs were taken 7 and 13 d after beginning the experiment.

Measuring skin pH and Bd infection The dorsal and ventral skin pH of *Rana muscosa* (n = 24) was measured with a flat glass probe. The intensity of infection measured in zoospore equivalents was determined by quantitative PCR according to Boyle et al. (2004). Both of these measurements were repeated 7 times at weekly intervals. We tested for a difference between the dorsal and ventral skin pH at the initial timepoint with a paired t-test, and for an overall correlation between infection intensity and ventral or dorsal skin pH by Pearson correlation. Since these frogs were all initially infected in the wild, no comparisons between infected and uninfected skin

pH or accurate analysis of skin pH through the time course of infection was possible.

DGGE Bacterial DNA was extracted from skin swabs using Qiagen DNEasy Blood and Tissue kit (Qiagen) and amplified with bacterial specific 16S rRNA gene primers 357F and 907R (Muyzer and Smalla 1998) designed to amplify the V4 and V5 regions of the 16S rRNA gene. Amplicons from PCR were analyzed by DGGE with a Bio-Rad D-Code System (Bio-Rad Laboratories) on 8% (w/v) polyacrylamide gels using gradients ranging from 30 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was carried out in 1 TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer at 70 V for 18 h at 60°C. Gels were stained with ethidium bromide (1:10 000 dilution) for 30 min and visualized on a UV transilluminator. Community profiles were aligned with pure isolates of Pedobacter cryoconitis for probable identification.

Treating adult *Lithobates pipiens* with itraconazole and heat therapy

Animal husbandry Adult *Lithobates pipiens* were purchased from a commercial supplier (J. M. Hazen Frog Company) and kept in groups until initiating the experiment. Frogs were placed into individual plastic enclosures (approximately 2 L) with access to water and were fed with crickets 2 to 3 times weekly after water changes. All frogs were weighed and swabbed for Bd at the beginning of the experiment and again on Day 17 after treatments and then changed to clean housing. Natural infections ranged from 0 to 4330 zoospore equivalents before beginning treatments.

Experimental design In February, 2007, 12 frogs were treated with itraconazole as above according to Nichols et al. (2000), except that daily treatments extended for only 5 d. Frogs were rinsed and placed into a new container after each treatment. A second treatment consisted of 10 frogs given heat therapy. Individual plastic enclosures containing frogs were placed in an incubator at 30°C overnight, and then the temperature was raised to 35°C for 24 h. The water was then changed, and frogs resumed feeding the next day. Control frogs were kept in larger plastic containers in groups of 3 and 4 individuals and kept at room temperature (about 23°C) without special treatment.

Treating newly metamorphosed *Limnodynastes peronii* with itraconazole

Animal husbandry Striped marsh frog *Limnodynastes peronii* larvae (n = 26) were collected from Tasmania's northwest coast, at sites where Bd-

infection status was unknown. Frog collections occurred in January and February 2009. Each animal was collected by hand using clean vinyl gloves, transferred to an individual plastic container (200 240 330 mm3) and transported to temperature (18 to 23°C) and light (12 h light:12 h dark) controlled facilities at the Department of Primary Industries, Parks, Water and the Environment (DPIPWE), Newtown Laboratories in Hobart, Tasmania, Australia. These individuals were originally collected for an exposure experiment. However, 12/26 (46%) of the newly metamorphosed *L. peronii* frogs died within 6 wk of metamorphosis, before the experiment had begun. Clinical signs were consistent with other studies reporting mortality due to chytridiomycosis occurring shortly after metamorphosis (Rachowicz and Vredenburg 2004, Garner et al. 2009a). Postmortem examinations on a subset of *L. peronii* metamorphs that died early determined that 4/5 (80%) of these individuals had Bd infections, one of which was confirmed with histopathology to be consistent with cases of severe chytridiomycosis (Berger et al. 1998, Pessier et al. 1999, see Fig. 5.4), and all had epidermal necrosis of an unknown etiology.

Experimental design We assumed that all metamorphs were infected with Bd; however, this could not be confirmed with PCR testing at the time. Metamorphs were monitored closely for clinical signs of chytridiomycosis. Two individuals displayed clinical signs of severe chytridiomycosis (loss of righting reflex, irregular skin sloughing and epidermal erythmia; see Fig. 5.4) and were therefore included in the treatment group. The remaining metamorphs (n = 12) were randomly assigned to control and treatment groups. Froglets were placed in round (5.5 cm diameter) clean plastic containers. Itraconazole (1%: 10 mg mL^{-1} Sporonox oral solution; Symbion) was diluted to 0.1% in 0.6% saline solution for a final concentration of 100 mg L^{-1} (Nichols and Lamiranda, 2000). Treated froglets were exposed to a bath of itraconazole solution (10 ml) for 5 min for 3 consecutive days before treatment was discontinued because of high mortality. Control froglets were exposed to an identical solution with no itraconazole. At the end of the 5 min period, the metamorphs were rinsed with tap water and returned to their individual containers with fresh tap water.

Treating larval *Alytes obstetricans* with commercial antifungals

Animal husbandry Tadpoles of *Alytes obstetricans* (n = 77) were collected in November 2009 (33 of them in Itingen, Switzerland (47° 27' 35.17" N, 7° 47' 2.12" E, 410 m above sea level [masl]) and 44 in Zunzgen, Switzerland (47° 26' 5.44" N, 7° 47' 56.73" E, 480 masl). For a separate experiment, Bd-infected tadpoles were captured from Zunzgen, and 89 were raised through metamorphosis between summer 2010 and spring 2011. Tadpoles

were reared in the laboratory in 0.28 m^2 plastic containers containing 80 L of tap water. The room was equipped with full-spectrum sunlight lamps on a 12 h photoperiod. Temperature was kept at 18 to 20°C. Water was changed once a week, and tadpoles were fed ad libitum with fish feed (Sera Spirulina Tabs, Sera GmbH). From January 2010, tadpoles were kept individually in 1.5 L plastic tubs using the same husbandry care. In the week before the experiment, we recorded the mass (Scaletec Instruments), the length from the snout to the beginning of the tail muscle, the developmental stage (Gosner, 1960), and we swabbed the mouthpart of the tadpoles with a sterile rayon-tipped plain swab with a plastic applicator (Copan). We used separate latex gloves for handling each tadpole to avoid cross contamination. These measurements were repeated after the experiments. We analyzed the swabs for the presence of Bd using qPCR following the protocol by Boyle et al. (2004). We ran samples in duplicate and repeated the analysis when the 2 results were inconsistent. For statistical analyses, we used counts of genomic equivalents (zoospore counts) to compare effectiveness of agents. For each individual the experiment was finished after a week of treatment. After the experiment, all tadpoles were successfully treated for 7 d with itraconazole according to Garner et al. (2009a) and brought back to their original pond according to per- mit specifications. Additionally, 89 metamorphs were treated with itraconazole diluted to 0.01% aqueous solution (10 mg L^{-1} ; (Garner et al., 2009a; Tamukai et al., 2011), for 3 consecutive days before treatment was discontinued because of high mortality. The surviving frogs were returned near their pond of origin.

Experimental design Tadpoles were randomly allocated to 3 treatments, each testing a different agent for efficiency against Bd. One tested agent was PIP Pond Plus (Chrisal). PIP Pond Plus contains a mixture of undefined probiotic bacteria (*Bacillus sp.*), enzymes, and 0.5 to 2.4% isopropanol. However, the exact composition of the agent is a corporate secret of Chrisal. To be sure that we were not erroneously measuring the effect of isopropanol alone, we conducted a small pilot study in which an equivalent dosage of isopropanol was added. No effect of isopropanol on Bd loads or on tadpole condition was detected. The second agent tested was Steriplant N (Swiss Steriplant AG). Steriplant N is electrochemically activated water. It contains 99.96% water (drinking water quality) and 0.04% oxidants (NaOCl-, ClO2, NaClO3, O3). Disinfection is thought to work through oxidation of microbes. The third tested agent was mandipropamid (Syngenta Crop Protection AG). Mandipropamid was developed by the derivatization of phenylglycinamides and mandelamides (Lamberth et al. 2008). It was developed as an effective agent against oomycetes. Mandipropamid is soluble in water up to a concentration of 4.2 mg L^{-1} .

(1) Efficiency test with PIP Pond Plus: infected tadpoles (n = 28) were

assigned to 3 different treatment groups and a control group (n = 7 in each group, housed individually). The agent was mixed into the water (1 L) daily over a period of 7 d. Depending on the treatment group the following dosages were applied: 0 μL (control, only stirring the water), 25, 50, and 100 μL. After 7 d the water was changed, and tadpoles were individually housed 7 d without treatment but with 2 water changes before swabbing them.

(2) Efficiency test with Steriplan N: infected tadpoles (n = 28) were assigned to 3 different treatment groups and a control group (n = 7 in each group, housed individually). We added 5 ml of the agent daily into the water (5 ml into 1 L = 5 ppm daily). Depending on the treatment group this treatment was applied 0 d (control, 0 ppm), 1 d (5 ppm), 2 d (10 ppm), or 3 d (15 ppm). After 7 d, the water was changed. Tadpoles were swabbed after 7 additional days of individual housing with 2 water changes.

(3) Efficiency test with mandipropamid: infected tadpoles (n = 21) were treated with different dosages of mandipropamid mixing different amounts of the agent into the water. The following dosages were tested: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, and 4 mg L^{-1} , and 3 controls. As mandipropamid is hardly soluble in water, we dissolved it in acetone (300 g L^{-1}) before applying it to the water. Hence, we tested 4 tadpoles with different dosages of acetone that were equivalent to the 0.01, 0.1, 1, and 4 mg dosages (not shown in the figure). Standard statistical analyses were done using R 2.10.1 for all experiments.

5.3 Results

Treating newly metamorphosed *Bufo bufo* with antimicrobial peptides

Survival and infection status The rationale for this experiment was to determine whether bath exposure to amphibian antimicrobial peptides would significantly reduce the level of detectable Bd infection in a susceptible species. Bd exposure reduced survival compared to unexposed toadlets 5.1(A).

Initial mass was a significant covariate with larger toadlets surviving longer (Cox regression, $p < 0.001$). Survival of toadlets in each treatment for the 35 d experiment was as follows. In Treatment 1 (unexposed controls), survival was 58.3% (mean: 27.3 d). In Treatment 2 (unexposed controls treated with peptides), survival was 63.6% (mean: 27.5 d). In Treatment 3 (Bd-exposed controls), survival was 17.6% (mean: 17.9 d). In Treatment 4 (exposed after prophylactic treatment with antimicrobial peptides), survival was 11.8% (mean: 15.6 d). In Treatment 5 (Bd exposed and treated with antimicrobial peptides 7 and 8 d after exposure), survival was 11.8% (mean: 20.9 d). Peptide treatment alone (Treatment 2) did not reduce survival

compared to unexposed controls (Treatment 1); however, both groups experienced approximately 40% mortality, typical for the species (Garner et al., 2009b; Loman and Madsen, 2010). Neither prophylactic (Treatment 4) nor remedial peptide treatments (Treatment 5) improved survival compared to infected controls (Treatment 3). However, of the surviving toadlets, 3 of 3 exposed controls were Bd positive at the end of the experiment, and 0 of 3 exposed toadlets that were treated with peptides were infected by the end of the experiment, indicating a potential benefit of peptide treatments (Fisher's exact test, $p = 0.05$). Thus, although treatment with peptides did not improve survival, it may have prevented or induced the clearance of infection in a small number of individuals.

Weight changes An analysis of variance of the rate of weight change throughout the experiment showed significant differences among treatments ($F = 3.632$, $df = 4$, $p = 0.012$). Unexposed toadlets had the highest rate of growth, peptide-treated toadlets showed intermediate growth rate, and Bd-exposed toadlets had the slowest rate of growth (Fig. 5.1B). Thus, treatment with *Pelophylax esculentus* peptides did not seem to negatively affect toad survival or growth; rather, peptide treatments before or after Bd exposure had similar beneficial effects on growth.

Treating adult *Rana muscosa* with itraconazole or biotherapy

Survival Bd infection loads on adult *Rana muscosa* indicated relatively heavy infections in all frogs at the beginning of the experiment (Fig. 5.2).

Thus, it was not possible to observe development of Bd infections in the frogs as in previous studies (Harris et al., 2009; Briggs et al., 2010), and mortalities began 3 d into the 51 d experiment. Survival of infected control frogs was 38.5% (mean: 31.5 d). Survival of infected control frogs was 38.5% (mean: 31.5 d). Survival of frogs treated with *Pedobacter cryoconitus* was 50.0% (mean: 38.5 d). Survival of frogs treated with itraconazole was 30.0% (mean: 25.4 d). A log-rank test on censored survival data did not demonstrate a significant difference among treatments ($\chi^2 = 1.494$, $df = 2$, $p = 0.474$; Fig. 5.2A). Controlling for initial frog mass and initial Bd load as covariates in a Cox regression did not show a significant effect of treatment ($p = 0.381$). All 17 frogs that died in either the infected control treatment or the biotherapy treatment were severely infected with Bd at death. Of the frogs that died in the itraconazole treatment, 2 of 7 were infected with Bd at death; thus, most frogs cleared infections before death. Three frogs survived the itraconazole treatment: Bd was initially undetectable from swabs on Day 60, but small zoospore loads were detected at a later time point, indicating incomplete clearance of Bd with itraconazole.

Weight changes Frogs in all 3 treatment groups lost weight. Pedobacter cryoconitis treatment reduced weight loss due to Bd infection compared to infected controls, and itraconazole-treated frogs had intermediate weight loss (ANOVA: F = 3.455, df = 2, p = 0.041; Fig. 5.2B). A general linear model did not indicate initial Bd load as a significant cofactor in weight loss (p ¿ 0.05).

Bd and skin pH Most frogs were heavily infected at the initiation of the experiment (Fig. 5.2B). Itraconazole quickly cleared Bd infections in surviving frogs, treatment with *Pedobacter cryoconitis* did not. Immediately after exposure to the bacterium (Day 2), zoospore loads were significantly reduced. At all later timepoints, infection intensity and prevalence were similar to values in infected controls (Fig. 5.2B). The skin pH of adult *Rana muscosa* naturally infected with Bd hovered around neutral (Fig. 5.3A), but the dorsal surface had a significantly lower pH than the ventral surface (paired t-test: $p < 0.0001$). Infection load was not significantly correlated with dorsal skin pH (Pearson's correlation: p = 0.542), but was weakly correlated with ventral skin pH (Pearson's correlation: p = 0.0056; Fig. 5.3B).

DGGE Microbial community analysis showed that 63% of *Rana muscosa* were naturally colonized by *Pedobacter cryoconitis* at the initiation of the experiment, and this did not significantly differ among treatments (Pearson $\chi^2 = 0.084$, df = 2, p = 0.959). One group of 20 frogs was given a bath treatment in the bacterium. At 7 d after treatment, the bacterium had not become established on previously *P. cryoconitis*-negative frogs, and the bacterium was cleared from 1 of 12 initially *P. cryoconitis*-positive frogs. After 13 d, 1 of 15 surviving frogs treated with *P. cryoconitis* retained the bacterium on its skin. This frog was not initially colonized. Of the surviving control frogs on Day 13 of the experiment, 0 of 9 showed *P. cryoconitis* colonization despite initial colonization on 6 of these frogs. Thus, this bacterium does not appear to be a good candidate for bioaugmentation because it does not persist after exposure of potential hosts.

Treating adult *Lithobates pipiens* with heat therapy in comparison with itraconazole

The short-term heat treatment was ineffective in clearing the infection in a small group of infected individuals, whereas itraconazole was effective in this experiment. Tab. 5.1 summarizes the results of diagnostic qPCR for each of the 3 treatments in comparison with untreated controls. Each treatment group included some frogs that were infected initially and some that were uninfected. All frogs had previously been exposed to infected conspecifics. The itraconazole treatment cleared infections for the 4 infected

frogs; however, 1 initially uninfected frog became Bd positive, and 1 frog died showing clinical signs of toxicity rather than acute chytridiomycosis. Heat therapy (24 h at 35°C) did not help clear infections. All control frogs kept in group enclosures were infected at the end of the experiment.

Table 5.1: *Lithobates pipiens*. Effects of itraconazole and elevated temperature on infection status of naturally infected adults. Bd: *Batrachochytrium dendrobatidis*.

Treatment	No. of frogs	Initial infection status	Treatment outcome
Itraconazole	4	Bd positive	4/4 lost infections
(100 mg L^{-1})	8	Bd negative	1/7 became infected, 1 death
Heat	4	Bd positive	4/4 lost infections
(35°C for 24 h)	6	Bd negative	1/6 became infected
Control	4	Bd positive	0/4 lost infections
(23°C)	3	Bd negative	3/3 became infected

Treating newly metamorphosed *Limnodynastes peronii* with itraconazole

The standard high-dose itraconazole treatment (100 mg L^{-1}) appeared to be highly toxic to these young metamorphs. Two froglets developed clinical signs of severe chytridiomycosis (Fig. 5.4) and died after 2 d of itraconazole treatment. Clinical signs of chytridiomycosis were noted in 2 additional treatment frogs on the same day. On the third day of treatment, we discovered that an additional 4 treatment and 2 control froglets had died. Treatments were discontinued at this point. The remaining froglets were sent to Lee Berger (James Cook University, Townsville, QLD, Australia) for isolation of Bd.

Treating larval *Alytes obstetricans* with commercial antifungals

Three commercial antifungals were tested on Bdinfected *Alytes obstetricans* tadpoles that had naturally acquired infections. There was no survival effect of any of the tested agents. The probiotic agent PIP Pond Plus was used in 3 different dosages with no significant effect on zoospore counts (Fig. 5A). Electrochemically activated water (Steriplant N) was used in 3 different dosages with no significant effect on zoospore counts (Fig. 5B). Doses of mandipropamid between 0.01 and 4 mg L^{-1} did not significantly affect

zoospore counts of infected A. obstetricans tadpoles (Fig. 5C). Infection prevalence effects are summarized in Tab. 5.2. We analyzed our data from the PIP Pond Plus treatment with different thresholds of zoospore equivalents to calculate infection prevalence (Fig. 5D). We analyzed the data with a factorial analysis of variance considering different thresholds factors and different treatments levels. We found that different thresholds (no threshold, 0.1, and 1 zoospore equivalent) produced different results (from 100% infection prevalence without a threshold to $< 60\%$ infection prevalence at a threshold of 1 zoospore equivalent, $p < 0.001$; Fig. 5D).

Table 5.2: *Alytes obstetricans*. Commercial antifungal treatments tested on infected tadpoles.

Treatment	**Summary**
PIP Pond Plus®	Infected *A. obstetricans* tadpoles (n = 28) were treated with 3 different dosages of the probiotic agent PIP Pond Plus®. All individuals but 1 were still infected at low levels after the treatment. No significant effect of the treatments on zoospore counts could be shown (Fig. 5.5 A).
Steriplant N	Infected *A. obstetricans* tadpoles (n = 28) were treated with 3 different dosages of electrochemically activated water (Steriplant N). All individuals were still infected after the treatment. No significant effect of the treatments on zoospore counts could be shown (Fig. 5.5 B).
Mandipropamid	Infected *A. obstetricans* tadpoles (n = 21) were treated with a range of dosages of mandipropamid. Five animals were cured afterwards: the 0.1, 1.4, and 1.6 mg l−1 and 2 animals from the control group. All other animals were still infected after the treatments. No significant effect of the treatment on *Bd* loads could be shown (Fig. 5.5 C).

Juvenile *Alytes obstetricans* were raised from naturally Bd-infected tadpoles in a separate experiment. They were never experimentally exposed to Bd in the laboratory. Collecting permits specified the return of these animals to the wild; thus, we began treatment with 0.01% itraconazole (10 mg L^{-1}) before release. Although the dose of itraconazole was reduced from the standard adult treatment dose, 10, 5, and 2 individuals out of the 89 metamorphs died after the first,second, and third days of treatment, respectively, and then treatment with itraconazole was stopped.

5.4 Discussion

Understanding the mechanisms by which chytridiomycosis can be suppressed is one of the first steps toward developing effective strategies to mitigate chytridiomycosis. Manipulation of temperature regime, microbiota, and antifungal compounds including peptides are all strategies based on a growing ecological understanding of the disease chytridiomycosis (Woodhams et al., 2011). Although the results of experimental treatments presented here do not demonstrate significant survival benefits, they will be instrumental in the development of effective treatment protocols. To briefly summarize the lessons learned. (1) Low doses of itraconazole or alternatives should be tested in future treatments of adult frogs to avoid lethal side-effects, and treatment should be applied early, before damage from disease accrues. (2) Antimicrobial peptide applications may reduce or eliminate Bd infection in hosts such as *Bufo bufo*, a species that does not produce skin defense peptides. (3) A minimum elevated temperature regime should be developed for a range of species and life stages. For example, a treatment period of longer than 24 h at temperatures between 30 and 35°C appears to be necessary. (4) The probiotic Pedobacter cryoconitis had beneficial effects on heavily infected *Rana muscosa*; however, the effects were transient (Fig. 5.2) because this bacterium did not persist on the host skin. (5) A commercially available probiotic treatment (PIP Pond Plus) added to water at recommended and higher doses against fish disease did not improve survival of infected *Alytes obstetricans* tadpoles either (Tab. 5.2, Fig. 5). In vitro sensitivity tests against Bd may reduce animal use for experimental treatments that are not known to be antifungal a priori. To date there are 2 published methods with apparent efficacy in clearing Bd infections in some species: antifungal drugs (itraconazole, voriconazole, terbinafine hydrochloride, and others) and temperature treatments (review in Berger et al. 2010, Bowerman et al. 2010, Martel et al. 2011, Woodhams et al. 2011). Itraconazole can be used to treat infected adults or larvae of some species; however, harmful side-effects including depigmentation of tadpoles have been noted (Garner et al. 2009). We showed here that lethal side-effects occur when using the commonly applied itraconazole treatment regimes at high or reduced doses (Nichols et al. 2000, Garner et al. 2009a) on adult *Rana muscosa*, *Alytes obstetricans*, and *Limnodynastes peronii*. Thus, we suggest testing of lower doses on a range of species and life-history stages with controlled pharmacokinetic safety trials as outlined by Berger et al. (2010). To date, few studies have examined the dose-related effects of itraconazole. Increasing evidence (review in Woodhams et al. 2011) indicates that elevated temperature can reduce the prevalence and intensity of Bd infections. Thus, elevated temperature may be used therapeutically for infected amphibians. However, the treatment regimes to minimize the temperatures and durations of treatment, in order to prevent unnecessary stress on the amphibians, has

not been systematically tested. Our preliminary experiment with Lithobates pipiens adults was not a systematic test of heat treatment, but it did indicate that temperature treatments longer than 24 h may be needed to clear Bd infection. If the zoospore stage is primarily affected by temperature or other treatments, then treatment regimes should continue throughout the life cycle of the pathogen. A life cycle is about 4 to 5 d in laboratory culture, but could be longer in amphibian skin or at suboptimal temperatures. A 10 d treatment at 30°C was more successful in the clearance of Bd in Rana catesbeiana and Acris crepitans adults (Chatfield and Richards-Zawacki 2011). A concern with the use of high temperature is that it may impair reproductive success. Examination of heat-treatment effects on egg and sperm quality should precede recommended protocols for elevated temperature.

In addition to these more traditional treatments, we provide results from alternative treatments, such as the application of beneficial microbes or antimicrobial skin peptides. Recent studies have demonstrated that biotherapy can effectively prevent the establishment of Bd and increase survival of mountain yellowlegged frogs *Rana muscosa* (Harris et al. 2009). In our experiment, treated frogs initially limited Bd proliferation and slowed weight loss compared to Bd-infected controls. DGGE microbial community analysis showed that although 63% (27/43) of R. muscosa were naturally colonized by Pedobacter cryoconitis, even after an additional bath treatment in the bacterium, only 1 of 15 frogs retained the bacterium on their skin 13 d post-treatment with individual housing in clean laboratory conditions. Although anti-Bd microbiota were previously shown to provide prophylactic benefits to R. muscosa and other amphibians (Harris et al. 2009, Becker and Harris 2010), treatment of heavily infected frogs may require more frequent antifungal treatment or more persistent and strongly protective skin microbes. Probiotic treatments of human and veterinary diseases, as well as applications in agriculture and aquaculture, are often applied continuously or in pulses for the greatest benefit (Yang et al., 2006; Nikoskelainen et al., 2003; Frohmader et al., 2010; Magnadottir, 2010; Thomas et al., 2010). Thus, a future research focus on prophylactic rather than remedial disease treatment may be warranted. Alternatively, more natural conditions, including housing frogs in groups in terrariums with soil may increase bacterial persistence. Since a reduction of microbial diversity may be a function of time in captivity, future trials should avoid this potential pitfall by using more natural conditions such as outdoor mesocosms. Microbes compete for resources and may alter the micro-environment indirectly through triggering host immune defences or directly by oxygen and nutrient utilization, biofilm formation, or by production of antagonistic substances or altering skin pH (Wilson 2005). Here, we show that the Bd infection load was weakly correlated with ventral skin pH in *Rana muscosa* (Fig. 5.3). This could be the result of immunopathology. Bd prefers a pH of 6 to 7, but growth is dramatically reduced at a of pH 4 to 5 or pH 8 (Piotrowski et al. 2004). Secretion of cationic

antimicrobial peptides may decrease the pH of the skin to create a hostile micro-environment. Lower pH on the dorsal surface, particularly during peptide discharge, may be a factor limiting infection there (Weldon and Du Preez 2006, North and Alford 2008, Sheafor et al. 2008). Here, higher pH on the ventral surface with higher Bd loads may be a cause or consequence of infection. Future studies on the effects of pH on skin infections may prove useful for disease management. We make the following recommendations: (1) Develop additional animal models. The experiments with *Bufo bufo* illustrate the potential for this model system in understanding the role of antimicrobial skin defenses against Bd. A recurring question is whether antimicrobial peptides on amphibian skin act as a protective mantle to resist infection, or as a first-aid kit to help deal with infection. Although we do not yet have a clear answer, ongoing studies suggest that some species are better protected than others by the constitutive release of antimicrobial peptides into the skin mucus. In our experiment, antimicrobial peptides may have prevented or eliminated Bd infection in 3 of 3 surviving toadlets that were exposed to Bd. One drawback to experiments with B. bufo metamorphs is their typically low survival under seemingly benign laboratory and natural conditions (Garner et al., 2009b; Loman and Madsen, 2010). (2) Attention to quality control of experimental protocols. Protocols to detect Bd infection in field surveys are often conservative to avoid false positives by using a qPCR detection threshold of 1 zoospore equivalent (Kriger et al. 2007, Vredenburg et al. 2010). In contrast, experimental treatments of Bd-in fected animals require protocols that are conservative to avoid false negatives. Thus, we do not recommend using a qPCR threshold infection intensity to dia gnose infection. Rather, here, we compare zoo spore equivalents among treatment and control groups to determine treatment effects on infection because estimates of in fection prevalence can be misleading. Working with prevalence requires the arbitrary definition of a threshold of zoospore equivalents above which an animal is considered infected or below which an animal is considered uninfected. This can generate inaccuracies when a treatment reduces Bd loads strongly but does not completely clear Bd infection. After such a treatment many animals could have zoospore counts below the threshold and hence be considered uninfected. Yet these could be false negatives. Communicating these results in terms of prevalence might give a very promising im pression of the treatment, but, in fact, all animals might still be infected at a low level. Our analysis of the PIP Pond Plus treatment with different zoospore equivalent thresholds illustrates this problem (Fig. 5D). A second recommendation, to reiterate Hyatt et al. (2007), is to swab treated animals at least twice after completion of the treatment, several weeks apart, to be sure that the infection was cleared. A third consideration is group size. Individual housing of amphibians may be important. In our preliminary experiment with *Lithobates pipiens*, transmission of Bd from infected to uninfected control frogs housed together emphasizes the importance of

individual housing unless several groups are used with group as the level of replicate to avoid pseudo-replication. However, group housing may stabilize infection status (if microbial populations fluctuate on individuals and re-colonization can occur via trans mission from group members) and be more indicative of disease dynamics in natural settings for some systems. (3) Share information with a public database. We are currently developing a public database of amphibian disease treatments that will incorporate both published and unpublished accounts of such treatments. We welcome contributions and suggestions. One portion of the database will be de dicated to microbes detected on the skin of various amphibians, including the methods of identification and results of growth-inhibitory activity of the microbes or their metabolites. Such a database, in addition to the published literature on primarily successful disease treatments, will be valuable to inform future research and to alleviate the threat of chytridiomycosis.

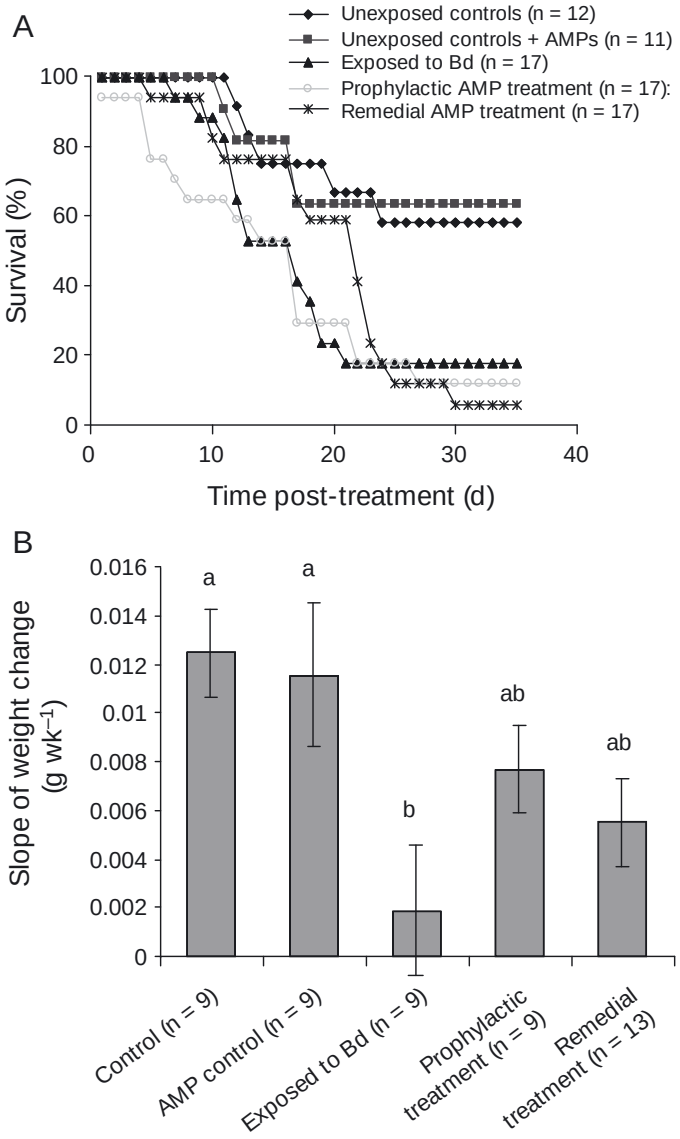

Figure 5.1: *Bufo bufo*. Newly metamorphosed *B. bufo* treated with antimicrobial peptides (AMPs) either before exposure to *Batrachochytrium dendrobatidis* (Bd) or after experimental infection. Peptides were harvested non-destructively from the skin of the chytridiomycosis-resistant species *Pelophylax esculentus* (see Materials and methods'). (A) Kaplan-Meier survival curve of toads in each treatment throughout the experiment (log-rank test on censored survival data: $\chi^2 = 15.179$, df = 2, p = 0.004). (B) Mean (±SE) change in weight throughout the experiment in all frogs weighed at least twice (ANOVA: F = 3.632, df = 4, p = 0.012). Identical letters above bars indicate homogeneous subsets (Tukey test).

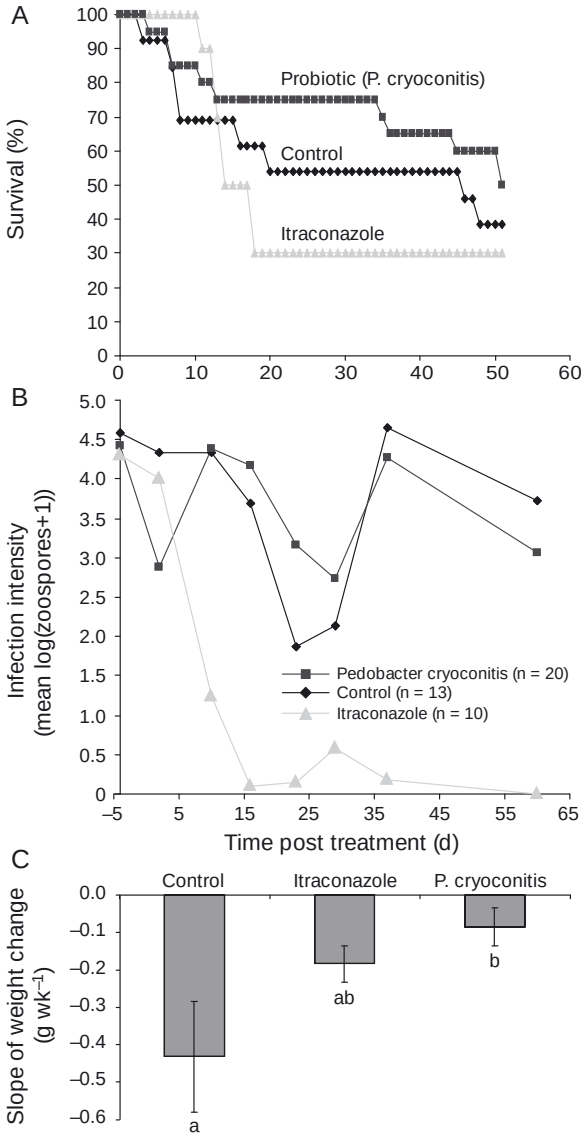

Figure 5.2: *Rana muscosa.* Results of treating adult *R. muscosa* naturally infected with *Batrachochytrium dendrobatidis* (Bd) with the antifungal itraconazole or the probiotic bacterium *Pedobacter cryoconitis.* (A) Kaplan-Meier survival curve of frogs after treatments (log-rank test on censored survival data: $\chi^2 = 1.494$, df = 2, p = 0.474). (B) Intensity of infection with Bd throughout the experiment. (C) Mean (±SE) change in weight throughout the experiment in all frogs weighed at least twice (ANOVA: F = 3.455, df = 2, p = 0.041). Identical letters below bars indicate homogeneous subsets (Tukey test).

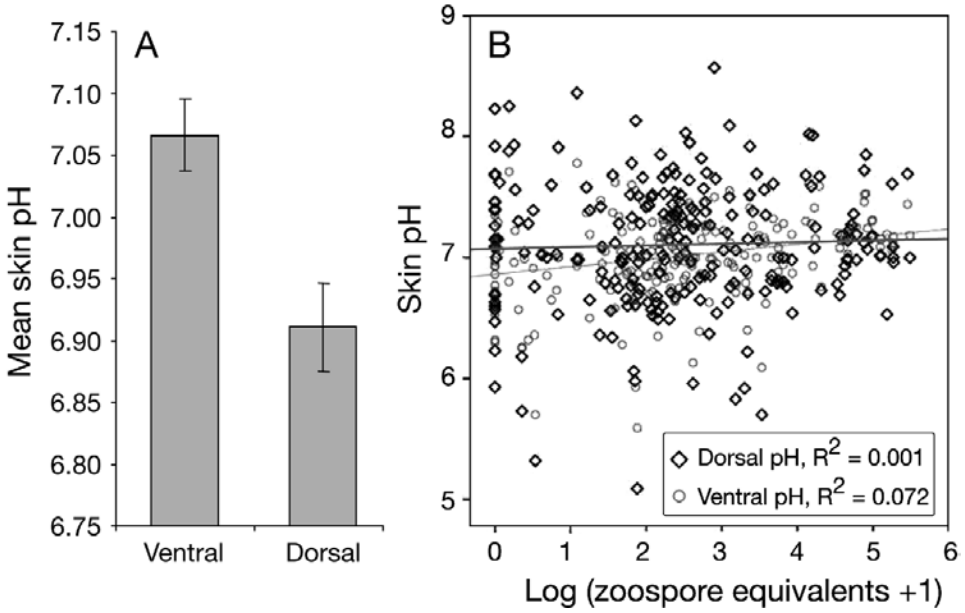

Figure 5.3: *Rana muscosa*. Skin pH of *R. muscosa* infected with *Batrachochytrium dendrobatidis* (Bd). (A) Mean (±SE) pH of ventral and dorsal skin surfaces. (B) Infection intensity shows a slight but significant correlation with ventral skin pH (light gray line) but not dorsal skin pH (dark gray line).

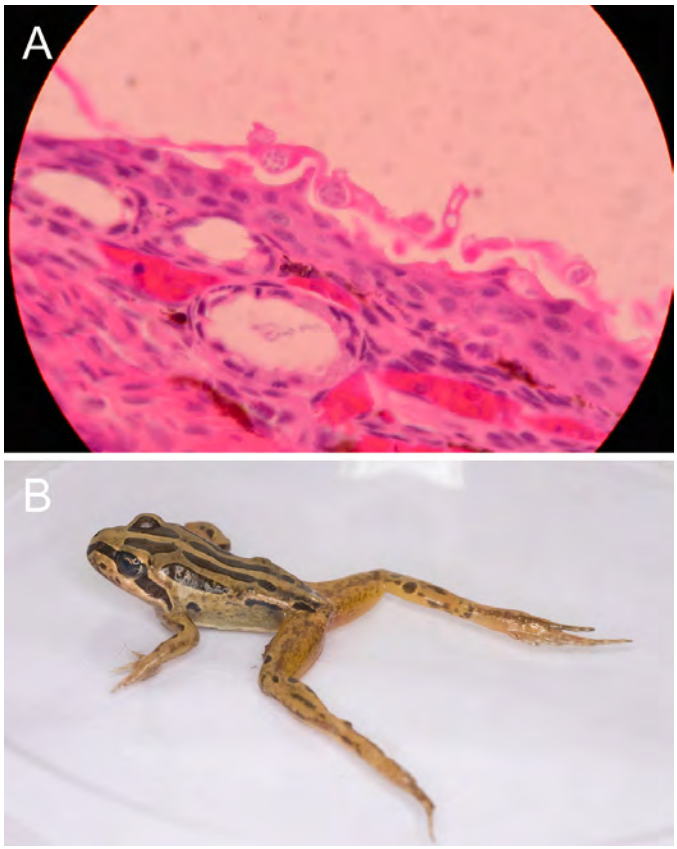

Figure 5.4: *Limnodynastes peronii*. Striped march frogs that had naturally acquired infections with *Batrachochytrium dendrobatidis* (Bd) as tadpoles remained infected upon metamorphosis. (A) A histological section of the skin of an infected *L. peronii* stained with hematoxalin and eosin, showing clear hyperkeratosis of the epidermis and typical Bd zoosporangia. (B) A newly metamorphosed *L. peronii* with clinical signs of chytridiomycosis.

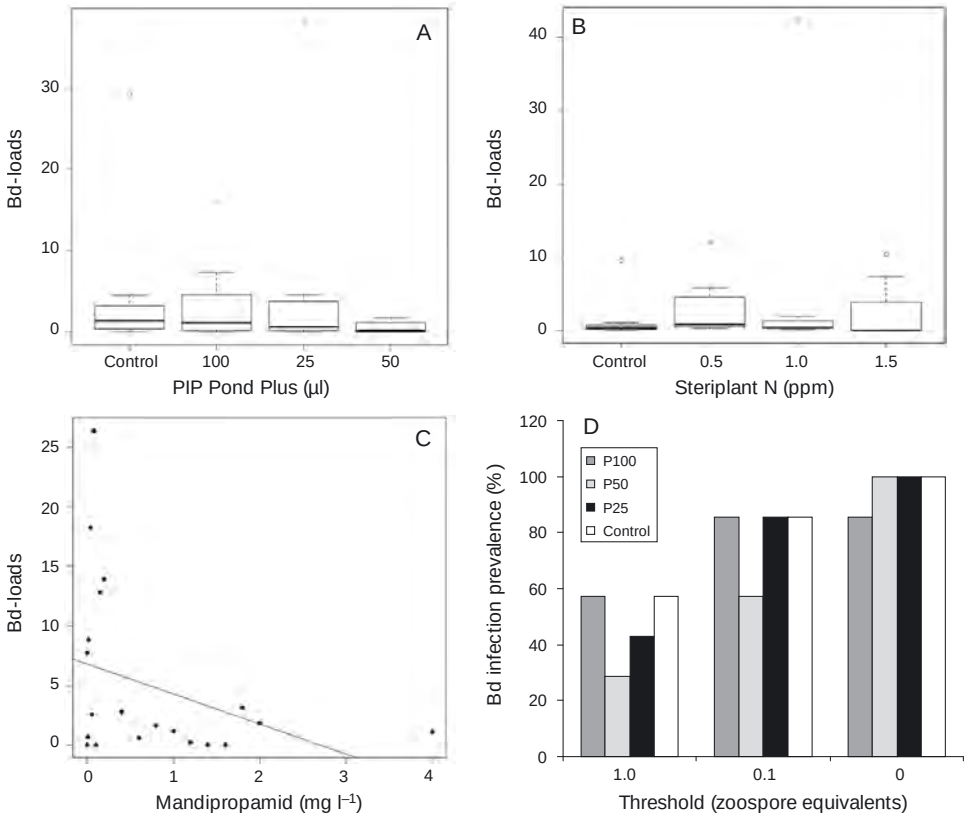

Figure 5.5: *Alytes obstericans.* The effects of 3 commercial antifungal treatments applied to larval A. obstericans on *Batrachochytrium dendrobatidis* (Bd) infection intensities (Bd load = log zoospore equivalents. (A) No significant effect of the PIP Pond Plus® treatment on Bd load (ANOVA: F = 0.7148, df = 24, p = 0.5528). (B) No significant effect of the Steriplant N treatment on Bd load (ANOVA: F = 0.3056, df = 24, p = 0.8210). Box plots show the medium value (line), 25 and 75% quantiles (box), 5 and 95% quantiles (whiskers), and outliers (°). (C) No significant effect of the mandipropamid treatment on the Bd load (linear regression: p = 0.0638). Five animals cleared infection, including 2 in the control group. The tadpole with the highest possible water-soluble dosage (4 mg L^{-1}) was still infected with Bd. (D) Thresholds of Bd zoospore intensity affect the interpretation of treatment results. All individuals with zoospore counts smaller than the threshold are considered uninfected. When no threshold is employed, almost all frogs regardless of treatment are considered infected. At higher thresholds, treatment seems to have a larger effect. Here, Treatments P100, P50, and P25 represent different dosages of the agent PIP Pond Plus® (see Treating *Alytes obstericans* with commercial antifungals').

Chapter 6

Accumulation of *Batrachochytrium dendrobatidis* and bacteria on the regressing tail of metamorphosing individuals

(Submitted to Amphibia-Reptilia)

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Abstract *Batrachochytrium dendrobatidis* (Bd) is a fungus infecting the skin of amphibians. On metamorphosing animals infection is difficult to detect because of the limited information concerning the location of Bd on the animals during this stage. Histological investigation revealed that Bd and bacteria accumulate on the reabsorbing tail of metamorphosing animals.

Keywords amphibian, chytridiomycosis, disease, tadpole

6.1 Introduction

Batrachochytrium dendrobatidis (hereafter Bd) is a fungus that infects the skin of amphibians. Bd is a generalist pathogen that has contributed to amphibian declines and extinctions worldwide (Stuart et al., 2004; Fisher et al., 2009). Bd was discovered in 1997 (Berger et al., 1998) and formally described in 1999 (Longcore et al., 1999). Bd causes the disease chytridiomycosis (Nichols et al., 2001; Voyles et al., 2009). Occurrence of Bd in the

amphibian host is highly associated with the keratinized cells of the epidermis of the skin (Berger et al., 2005b). The distribution of keratinized cells changes according to the life stage of the amphibian and infection with Bd usually parallels these changes (Marantelli et al., 2004). During the tadpole life-stage, keratin is usually found only on the mouthparts. At metamorphosis the keratinized mouthparts are shed before keratin is layered on the skin of the entire body (Marantelli et al., 2004). Hence, detecting Bd on a metamorphosing animal can be challenging since the dynamic of the tissue changes occurring in the skin might influence the attachment and distribution of Bd (Marantelli et al., 2004). Here we report the results of a histological study which shows that Bd and bacteria can accumulate also on the reabsorbing tail of metamorphosing midwife toads (*Alytes obstetricans*).

6.2 Material and Methods

The detection of the accumulation of Bd on the regressing tail was observed in the context of a larger experiment on Bd mitigation (Woodhams et al. 2011; C. C. Geiger et al., unpublished manuscript). Part of this experiment was a histological investigation on metamorphosing individuals of the midwife toad *Alytes obstetricans* aiming to evaluate the spectrum of changes which different antifungal compounds might have induced on the tissues of the toads. Seventy tadpoles of *Alytes obstetricans* naturally infected with Bd were caught in early spring in Zunzgen, a site located in the canton Baselland in northwestern Switzerland (7.801185°E, 47.436677°N, 480 masl) for the experiment. Tadpoles were raised in 10 outdoor mesocosms, all of them containing 650 L of water, leaf litter, plankton and a self-sustaining aquatic community composed of zooplankton and microorganisms (Wilbur, 1987). Upon metamorphosis the tadpoles were caught and housed individually indoors in clear plastic containers, containing leaf litter and some water. Containers were tilted so that water and land were both available. The room was equipped with full spectrum sunlight lamps set for a 12 h day length. Room temperature was maintained at 19 - 21°C. Once the toadlets reached Gosner stage 44 (Gosner, 1960), 11 individuals were randomly selected and euthanized with MS 222 that was buffered to pH 7. The tadpoles were fixed in 10% buffered formalin and sectioned cranio-caudally along the midline. The two symmetrical portions of the body were then layered flat in the histocassette, processed as such and embedded in paraffin. Five micron-thick sections were obtained from each specimen and stained routinely with hematoxylin and eosion (H&E). All the sections obtained were screened before full histological examination and additional deep cuts were performed until the major organs were visible in the examined sections, if necessary. Additionally, special stains including Grocott, Gram, PAS, and Zihel-Neelsen were applied as appropriate. Slides were observed under light microscopy

by a single veterinary pathologist and the detected integument changes were recorded for each specimen. Abundance of Bd zoosporangia was ranked according to a subjective scoring system into five categories (0, 1, 2, 3, 4; see Fig. 6.1).

6.3 Results

We found variable thickening of the epidermal layer (hyperplasia) with hyperkeratosis (parakeratotic). Presence of Bd was variable, from very few fungal organisms to myriads forming thick "pseudo-membranes" with the superficial devitalized layers of the epidermis (Fig. 6.1). While we observed variable amounts of Bd on most body parts, the most severe skin lesions, almost invariably associated with accumulation of Bd, were observed more frequently in the caudal portion of the body, and almost regularly occurring in correspondence of the "wrinkling" and "bending" of the epidermis of the regressing tail. Significant collections of bacteria were also observed in correspondence with the most severe skin lesions. More specifically, six out of the 11 individuals from the control group showed more severe lesions in the caudal part of the body. Five of the six animals with more severe lesions on the tail showed a correspondent higher amount of Bd on the tail. For one animal, despite the most severe lesion was observed on the tail, a higher number of fungal organisms was observed on the caudal aspect of the abdominal skin.

6.4 Discussion

We do not know why Bd and bacteria accumulate on the regressing tail and what the consequences of this accumulation might be (e.g. for host-pathogen interactions). Marantelli et al. (2004) suggested that there are accumulations of sloughing keratin on the degenerating epidermis of the tail. It is possible that the accumulation of Bd in this area of the skin might be secondary to the indirect formation of recesses in the integument of the regressing tail providing ideal "collection sites" for the fungus to attach and grow. Here, Bd would be less likely to undergo easy mechanical removal. Considering that the life cycle of Bd takes 4 to 5 days (Berger et al., 2005a) and the re-absorption of the tail takes between 3 and 6 days (Walsh et al., 2008), it is consequential that Bd can persist at least for one but probably for several generations on the regressing tail. Accumulation of Bd on the regressing tail may allow Bd to persist through host metamorphosis.

The tail would then seem to be a part of the body where Bd likely occurs during metamorphosis. This was previously reported by Marantelli et al. (2004) for the Australian frog Myxophyes fasciolatus and we confirm the pattern for the European toad *Alytes obstetricans*. The detection of Bd on

metamorphosing amphibians could then benefit from this observation. For studies concerning the epidemiology and disease ecology of Bd, we therefore suggest to swab not only feet and belly of metamorphosing anurans (Garner et al., 2009) but also the tail. By doing so, we believe that the number of false negatives can be reduced.

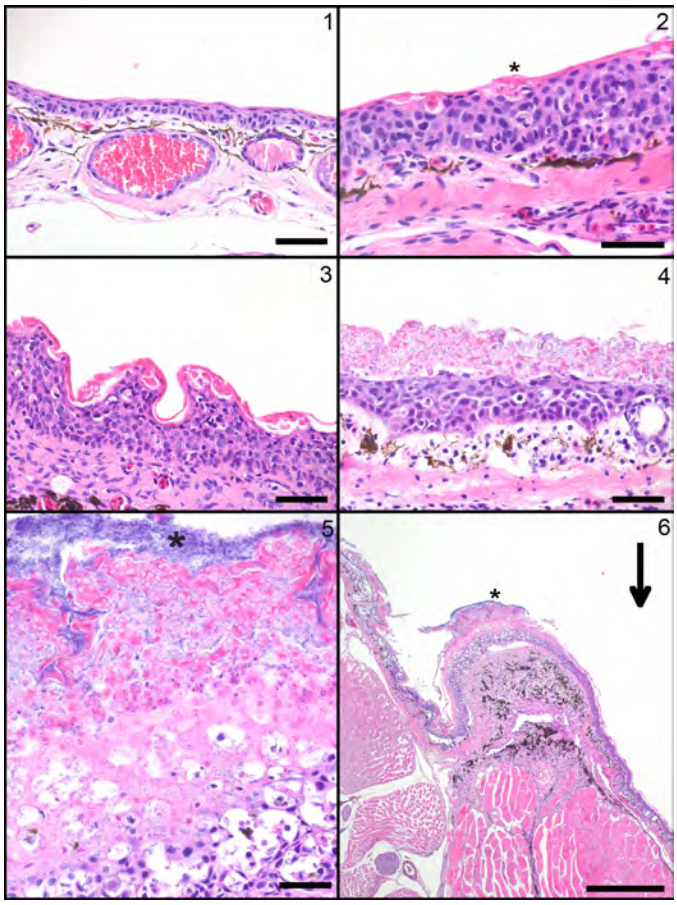

Figure 6.1: (1) Skin. A histological section of normal skin from *Alytes obstetricans* is shown. Scoring for presence of Bd=0. Size bar= 50 μm (H&E stain). (2) Skin. There is patchy collection of fungi (asterisk) within the keratin layer of the epidermis, which is also mildly thickened. The epidermis is thickened (hyperplasia) and infiltrated by few numbers of mononuclear cells. Scoring for presence of Bd=1. Size bar= 50 μm (H&E stain). (3) Skin. There is more pronounced hyperkeratosis (parakeratotic) than that observed in (2) with more abundant fungal organisms, piling up to 3 layer thick. There is mild to moderate hyperplasia of the epidermis with vacuolization and infiltration by mononuclear cells. Scoring for presence of Bd=2. Size bar= 50 μm (H&E stain). (4) Skin. A thick mat of fungal organisms is embedded within the thickened keratin layer of the epidermis. Bd piles up to 12 layer thick. Mild infiltration of mononuclear cells is observed in the hyperplastic epidermis. Scoring for presence of Bd=3. Size bar= 50 μm (H&E stain). (5) Skin. A very thick layer of fungal organisms admixed with bacteria (asterisk) is overlaying the necrotic and degenerating epidermis. Scoring for presence of Bd=4. Size bar= 50 μm (H&E stain). (6) Regressing tail. A thick mat of fungal organisms is present over the stump of the regressing tail (asterisk). Other less prominent but abundant collections of Bd are seen in the clefts of the bending epidermis. Size bar= 1mm; the arrow shows the orientation of the body (H&E stain).

General Discussion

Amphibians are more threatened than any other vertebrate taxon (Stuart et al. 2004, Hoffmann et al. 2010). Reasons for this threat are mainly overutilization and habitat loss (Stuart et al., 2004). Only recently a new severe threat to amphibians appeared which contributes to population declines and drives species to extinction: the emerging infectious disease chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd) ((Berger et al., 1998; Daszak et al., 2000; Stuart et al., 2004; Skerratt et al., 2007). Hence, successful amphibian conservation depends beside habitat restoration and reduction of amphibian exploitation also on effective mitigation of chytridiomycosis.

While it is in principle easy to reduce population declines due to habitat loss, habitat fragmentation or traffic, reduction of disease induced threats is a challenging issue. Problems might be centered around obstacles concerning politics and law, lack of knowledge about many diseases of wildlife, the absence of basic data on wildlife populations, difficulties with monitoring, as well as logistical limitations ((Keesing et al., 2006). In the search for a mitigation strategy against chytridiomycosis one way could be to build on the experiences that were made during the mitigation of other infectious diseases. This approach might work in some cases however as mitigation of wildlife diseases is a new topic in species conservation we face new problems and might need new solutions for them.

A glance at the mitigation methods and experiences in the context of infectious diseases in human and life stock reveals that mitigating infectious diseases is a difficult endeavor. Among all diseases ever recorded on this planet there are only two where a global eradication was successful: 1980 the smallpox were declared eradicated and 2011 the rinderpest. The key to success was in both cases a large-scale vaccination program (Mayr, 2003; Mariner et al., 2012).

There are other cases where disease mitigation was to a greater extent successful but the pathogens got reintroduced into previously disease-free areas (Fevre et al., 2006). This was the case for rabies, which was mitigated by culling and oral vaccination programs, for bovine tuberculosis where the spread of disease could be predicted through knowledge of the network among cattle dealers. It was the case for trypanosomiasis in domestic

animals and humans where the disease was managed in an integrated way by treatment of animals to prevent disease spread to humans. Mitigation of the foot and mouth disease was as well to a large extent successful where sources of infection were identified through DNA sequencing techniques so that these sources could have been eliminated quickly (Fevre et al., 2006).

Mitigation of other infectious diseases proved to be very difficult and was less successful for multiple reasons. This relates for instance to the influenza virus, AIDS, cholera or malaria. The methods used to control these diseases involve vaccination, reduction of transmission, treatment of diseased patients, early diagnosis, prevention programs and communication of mitigation strategies (Inglesby et al., 2006; Mukherjee, 2007; Woodborne et al., 2008; IWMI, 2010). Problems mainly arise thorough lack of money, rapidity of spread, inaccessibility to vaccines or drugs, structural violence, resistances and lack of knowledge about how the disease is embedded into the environment (Inglesby et al., 2006; Mukherjee, 2007; Woodborne et al., 2008; IWMI, 2010).

Similar difficulties have also been experienced regarding mitigation of infectious diseases in agriculture. Mitigation of rapidly spreading fungal diseases known as wheat rusts are so far limited to fungicide sprays to which resistance may evolve (Hovmoller et al., 2010). The most promising approach in this case is currently the breeding of plants that are resistant to the fungus (Hovmoller et al., 2010). Other mitigation strategies that currently come into play are focused on the investigation of different pathogen strains to identify the geographic source of the pest or pathogen and reveal interactions with other pests, pathogens or environmental factors (Kim and Sappington, 2006; Martin et al., 2012).

Mirroring these strategies and experiences that exist in the context with human diseases and livestock and considering that we need a mitigation method against a wildlife disease to protect endangered species the following approaches emerged: One approach is to stop the human induced dissemination of Bd by controlling the world trade in amphibians (Fisher and Garner, 2007) or by developing standard biosafety protocols to avoid spreading Bd on a smaller scale (Schmidt et al., 2009b). This approach proved to be effective as we have seen it with bovine tuberculosis or foot and mouth disease. A second approach is the establishment of captive breeding programs. This approach is not a mitigation method but it preserves the most susceptible species from disease-induced extinction (Zippel et al., 2011). The third approach is the development of a mitigation strategy against Bd. In the context of the third approach different ideas were suggested or tested experimentally and valuable results were achieved. These ideas were to a certain degree borrowed from agriculture and from mitigation of human and livestock diseases but some of them are new.

One idea was to use amphibian skin microbiota and antimicrobial peptides with Bd inhibitory activity to combat chytridiomycosis (Woodhams

et al., 2011). Here, the most promising strategies are mitigation by introduction of bacteria into the soil which inhibits colonization by Bd on the skin of the amphibian (Muletz et al., 2012). Moreover bacteria and antimicrobial skin peptides were found to suppress disease in some amphibians (Garner et al., 2009b; Woodhams et al., 2011). A further idea was to find natural enemies of Bd or ecological conditions that are unfavorable for the pathogen (Woodhams et al., 2003). In this context treatments with elevated temperature were very successful. Keeping amphibians in elevated temperature has the potential to clear or drastically reduce infection burdens (Woodhams et al., 2011). Finally, an idea is borrowed from agriculture where fungal diseases of crops are treated using chemical antifungal agents (Woodhams et al., 2011). In this regard a very successful treatment was found by Garner et al. (2009a), who cleared tadpoles with the antifungal agent Itraconazole.

What has been missing until now is a strategy to mitigate Bd in natural habitats potentially including a method which not only reduces Bd infection but transmission of the pathogen from infected to non-infected individuals. Most of the mitigation methods that were developed so far were conducted in the laboratory and none of them proved to be an implementable and effective strategy to mitigate Bd in natural habitats. Some of these methods were simply not tested yet in natural habitats but have the potential to be very effective. Other strategies might not satisfy the demands of a mitigation method in natural habitats.

A mitigation method against Bd in natural habitats has to fulfill multiple objectives: most importantly it should reduce Bd infection and possibly Bd transmission not only under controlled conditions in the laboratory but importantly also in the complex framework of a natural ecosystem. Furthermore a treatment should be safe for individual amphibians as well as for non-target organisms in the environment. A treatment should also be simple to implement because it might be applied in remote areas. Finally, as we need to treat whole or parts of a population it should be possible to treat large numbers of amphibians. Hence, we need to explore new treatments that seem promising for use in natural populations.

This was exactly the goal of my dissertation: Developing methods to mitigate chytridiomycosis in natural habitats. The approach I persuaded was partly borrowed from agriculture by using antifungal agents for a treatment against Bd and partly I tested ecological conditions that are unfavorable for the pathogen.

In chapter 1 I describe an experiment in which an ecological condition was used as mitigation method against Bd. As Bd is sensitive to high temperature (Piotrowski et al., 2004), I exposed tadpoles of *Alytes obstetricans* to low, medium and high temperatures. I found that most tadpoles lost the infection, when they were kept for 5 days at temperatures higher than 26°C. When applying elevated temperature as treating method I recommend some acclimatization of the tadpoles before the treatment and a pilot study when

novel species are treated. As conservation measures I suggest to reduce canopy cover and to construct shallow water zones which results in the rise of temperatures in the ponds (Skelly et al., 2002) and perhaps prevalence will drop. Additionally warmer water speeds up larval development which implies that fewer tadpoles will hibernate (Thiesmeier, 1992) and therefore population growth rate will increase (Govindarajulu et al., 2005). Warmer ponds may thus boost population growth rate and reduce Bd prevalence.

In chapter 2 I used chemical agents to treat infected tadpoles against Bd. I kept tadpoles of Alytes obstetricans for seven days individually in solutions of the two chemical agents General Tonic® and Virkon Aquatic® . General Tonic® reduced Bd infection efficiently while Virkon Aquatic® showed no effect on Bd infections. Chapter 2 shows that chemical agents may be used to treat amphibian larvae against Bd infection. I found a side effect of General Tonic® on size of tadpoles and further experiments are necessary to test side effects of this agent on the pond ecosystem and also to check whether the side effect on tadpoles disappears in a more natural context.

In chapter 3 I set up semi-natural mesocosms to test for the effects of antifungal chemicals in a broader ecological context. I introduced mollusks, insects, plants, crustaceans, plankton and infected and uninfected tadpoles of *Alytes obstetricans* and uninfected tadpoles of other species into the mesocosms. I added three different chemical agents (General Tonic® , Virkon Aquatic® and PIP Pond Plus®) to the mesocosms. I checked for the efficiency of these compounds in reducing Bd infection of infected tadpoles and transmission from infected to uninfected tadpoles. Furthermore, I also tested for side effects of the antifungal treatment on amphibians, mollusks and on different responses of the ecosystem. PIP Pond Plus® neither had an effect on Bd infections, nor did I detect any side effects. General Tonic® reduced Bd infection and had some negative short-term effects on mollusks and on the structure of the ecosystem like plankton density, but the function of the ecosystem was unaffected returning no effects on litter breakdown. Virkon Aquatic® had no effect on Bd loads and Bd prevalence of infected individuals, but it blocked Bd transmission. Virkon Aquatic® caused no short-term side effects on the ecosystem, but in the long run ecosystem structure was affected by Virkon Aquatic® , mainly boosting snail reproduction and plankton numbers. However, the crucial question is: what are the costs and the benefits? While the benefit for amphibians seems clear, we know that there are costs but the costs are much harder to quantify. In such a situation, a precautionary approach is recommendet. That is, one should only use antifungal agents in natural habitats when the expected benefit is large and likely outweighs the costs. A benefit may be large if one is witnessing a catastrophic Bd-induced population decline. In situations, where Bd does not appear to have immediate catastrophic effects, e.g. Tobler et al. (2012), the use of antifungal agents might not be recommended. Moreover costs of antifungal treatments may be more admissible in simple habitat types like

the gorges inhabited by the Mallorcan midwife toad Alytes muletensis or man-made habitats such as gravel pits. In a next experiment I tested the treatment with antifungal agents in natural habitats.

In chapter 4 I report on three experiments in which I tested chemical antifungal treatments with Itraconazole and General Tonic® in mesocosms and in natural ponds. I did not add the agent directly to the water. Instead I caught tadpoles out and treated them in tanks next to the pond to avoid side effects on non-target organisms. After the treatment tadpoles were released into the pond. I did this procedure first in mesocosms using the chemical agent Itraconazole (Garner et al., 2009a; Tobler and Schmidt, 2010). Here I found that a treatment in the tadpole stage reduced Bd infection. Bd infection stayed on a low level throughout metamorphosis and reduced the mortality of metamorphosed individuals. Subsequently I tested this method in the second experiment in natural ponds by treating tadpoles once with the chemical agent Itraconazole; in a last experiment I treated tadpoles monthly with General Tonic® . Both experiments in ponds were followed by a monitoring of Bd infection over the period of one year. I found that treating tadpoles with chemical agents outside the pond and releasing them after the treatment reduced Bd infection in the short term. However the effect disappeared after two months. A part of the tadpoles were not caught out and thus not treated. These untreated tadpoles showed as well reduced Bd infection. Finally I found that Bd infection is lowest in fall. A treatment in fall might thus be a new approach in order to keep Bd infection in a low level. These results suggest that antifungal agents can reduce infection but cannot eradicate Bd from natural environments. However, treatments are effective for a short time and thus might serve as an emergency measure in the case of a Bd-induced mass die-off.

Chapter 5 is a conglomeration of treatments that were not considered as successful. The chapter includes treatments to treat Bd-infected frogs including *Alytes obstetricans* tadpoles and metamorphs, *Bufo bufo* and *Limnodynastes peronii* metamorphs, and *Lithobates pipiens* and *Rana muscosa* adults. The experimental treatments included commercial antifungal products (itraconazole, mandipropamid, steriplantN, and PIP Pond Plus®), antimicrobial skin peptides from the Bd-resistant *Pelophylax esculentus*, microbial treatments (*Pedobacter cryoconitis*), and heat therapy (35°C for 24 h). None of the new experimental treatments were considered successful in terms of improving survival; however, these results may advance future research by indicating the limits and potential of the various protocols. Learning from failed treatments' is essential because the time we lose by doing the same failures twice is valuable time we lose in the development of an urgently needed mitigation method against Bd.

Chapter 6 is a notice about the observation that Bd and bacteria accumulate on the reabsorbing tail of metamorphosing animals. So far it was difficult to detect Bd infection on metamorphosing individuals because

of lack of information about the position of Bd on the animal during this developmental stage. The notice in chapter 6 helps to find Bd also on metamorphosing individuals.

If we now go back to the mitigation methods that are available we can summarize that there are methods to treat infected individuals in the laboratory with skin microbiota, with antimicrobial peptides or with chemical agents. A promising strategy for treatments in natural habitats might be introduction of bacteria into the soil which inhibits colonization by Bd on the skin of the amphibian (Muletz et al., 2012) as well as elevated temperature. None of these methods was tested in the field. My dissertation provides methods of which we know how they work under controlled conditions in the laboratory, we know their effects on Bd infection and transmission and their side effects on non-target organisms under semi-natural conditions in mesocosms and we know how they work in the complex framework of a natural ecosystem.

A new and ecologically compatible approach might be to reduce canopy cover and to create shallow water zones in order to provide warmer water which may result in lower Bd prevalence and higher population growth rate. Another new finding was that transmission can be blocked by adding antifungal agents to the water. If we add such an agent when Bd prevalence is low, for instance when tadpoles hatch, might ensure that prevalence stays low. Further experiments should be done to find how long this treatment is effective.

New was the finding, that commercially available antifungal agents have the potential to reduce Bd loads and Bd prevalence under semi-natural conditions. Negative side effects on amphibians were, in my opinion, context- and species-specific but overall relatively benign. Yet, antifungal agents have a number of side effects on the ecosystem. I thus recommend chemical treatments of ponds exclusively if a catastrophic Bd-induced population decline is foreseeable and I also recommend to careful assess the costs and benefits of such a treatment in advance. In general I advise against adding the chemicals directly into the pond water because of side effects on the aquatic ecosystem. I rather recommend catching animals and treating them outside the pond in groups, at best shortly before metamorphosis because treating tadpoles is effective throughout metamorphosis. This leads us to a further new outcome of my dissertation: catching tadpoles out of a pond and treating them with a chemical agent in tanks next to the pond reduces not only Bd loads of the treated tadpoles but also prevalence of those tadpoles that remained in the pond and were never treated. In the course of the experiments in natural ponds I observed that Bd infection is lowest in fall. This is an interesting finding in the disease dynamics of Bd and help to define the right time for a treatment. A treatment in fall might help to keep Bd infection on a low level.

Future problems could arise through development of new and more vir-

ulent strains of Bd or through resistances of Bd against the treatments. Resistances however might also be developed among susceptible amphibian species to Bd. Researchers suggest that Bd-infection may initially cause a reduction in population abundance but thereafter, populations remain stationary or can even increase (Briggs et al., 2010; Tobler and Schmidt, 2010) implying that enzootic Bd not necessarily causes population declines.

The drawback of course is that Bd will persist and what we have in hand are some emergency strategies to confine the pathogen for a short term on a very limited area. What we can do is go on with research to find mitigation strategies that reduce Bd infection and transmission in whole populations, like blocking transmission, vaccination, culturing antimicrobial skin bacteria and importantly also more integrated strategies including habitat restoration. What remains finally is hope; the hope that our mitigation strategies help to avoid extinction of susceptible species so that we get more time to develop mitigation methods against Bd with permanent effects and one day, hopefully, chytridiomycosis disappears from the long list of threats to amphibians.

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Curriculum vitae

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Education

2009 - 2013	PhD student at the Department of Ecology, Institute of Evolutionary Biology and Environmental Studies, University of Zurich; Thesis title: Developing methods to mitigate chytridiomycosis, an emerging disease of amphibians
2004 - 2006	MSc studies in Zoology at the Department of Conservation Biology, Zoological Institute, University of Bern; Thesis title: Ecological requirements of the Alpine Salamander *Salamandra atra*: assessing the effects of current habitat structure and landscape dynamics on local distribution
2001 - 2004	Undergraduate Studies in Biology at the University of Bern
1994 – 2001	High School at the Kantonsschule in Chur, Type B (Latin)

Publications

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